

COMPLEMENT-MEDIATED
NEUTROPHIL ACTIVATION

by

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ABSTRACT

This study has examined activation of human polymorphonuclear leucocytes (neutrophils) by complement components and the role of such activation in the production of inflammation. Using release of the secondary granule component cobalamin binding protein as a marker of cell activation, complement activated serum was shown to contain a single low molecular weight peak of degranulating activity for cytochalasin B treated neutrophils dependent on the presence of C_5 .

Thus neutrophil degranulation could be used as a functional assay for the detection of C_5 fragments and anti- C_5 fragment activity.

Cobalamin binding protein was also slowly lost from cells in the presence of serum, a phenomenon which is not complement dependent but which may be of importance in control of granulopoiesis.

Autologous complement-activated serum, when injected intradermally rapidly produced an inflammatory response of flare, wheal and accumulations of intravascular neutrophils. The wheal and flare response was C_5 dependent and also partially neutrophil dependent, since severely neutropenic subjects showed significantly impaired skin responses, despite normal serum levels of C_5 fragments, and irrespective of platelet count.

Possible mechanisms of neutrophil involvement in this microvascular response to C_5 fragments were examined. Complement-activated neutrophils did not release the

inflammatory arachidonic acid products prostaglandin E_2 or leukotriene B_4 , nor were the skin responses to complement reduced by aspirin, hydrocortisone or ibuprofen. Oxygen radicals did not appear to be important as chronic granulomatous disease carriers (and one affected male) had normal reactions. Chronic granulocytic leukaemia patients, however, had impaired responses, for reasons as yet unclear.

Skin responses to complement in normal subjects were reduced by local anti-histamine drugs of anti- H_1 type. This could, however, have been due to neutrophil blockade rather than histamine antagonism, for H_1 antagonists significantly reduced neutrophil degranulation *in vitro*, as did histamine. Studies with H_1 and H_2 antagonists suggested the presence of H_2 receptors on neutrophils with H_1 antagonists having local anaesthetic type effects on the cell membrane.

Neutrophil degranulation was also blocked by extracts of the plant *Tanacetum parthenium* (feverfew), a herbal remedy for migraine and arthritis. Studies with different degranulating stimuli suggested that feverfew might be acting on the pathway to protein kinase C activation. Phagocytosis of opsonised yeasts was also blocked by feverfew.

Neutrophil activation by complement fragments thus appears to lead, by unknown mechanisms, to increased vascular permeability and vasodilatation. Such activation which may be particularly important in the pathogenesis of the adult respiratory distress syndrome and rheumatoid arthritis, appears to be amenable to pharmacological modulation by H_1 antagonists and feverfew extracts. The therapeutic significance of these observations remains to be tested.

DECLARATION

I hereby declare that this thesis has been composed entirely by myself, and that the studies contained herein have been conducted either solely by myself, or in collaboration with others. Such collaboration is acknowledged at the beginning of the relevant chapters. Where collaborative studies have been done, I have contributed significantly to the work.

July 1986

ETHICAL CONSIDERATIONS

All studies in this thesis involving human subjects, including blood sampling, skin testing and drug administration, have been performed with the approval of the Nottingham City Hospital Ethical Committee, and with the informed written consent of the subjects.

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I owe a special debt to the many volunteers and patients who showed such willingness to donate blood, and to take part

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PUBLICATIONS AND PRESENTATIONS AT MEETINGS

Parts of this thesis have already been presented and/or published as follows:-

Presentations at meetings

1. Release of vitamin B₁₂ binding protein from cytochalasin treated neutrophils - a functional assay for C_{5a} (oral presentation).

European Society for Clinical Investigation,
Milan, April 1984 (abstract in European Journal
of Clinical Investigation (1984) 14: A33)

2. The role of the neutrophil in the inflammatory response to intra-dermal zymosan-activated serum (poster).

3rd International Congress on Inflammation,
Paris, September 1984.

3. The role of the neutrophil in the inflammatory response to intra-dermal zymosan-activated serum (oral presentation).

British Society for Haematology Scientific Meeting,
London, November 1984.

(Awarded British Society for Haematology's Young
Investigator Award).

4. Neutrophil degranulation is inhibited by anti-histamine drugs of anti-H₁ type (oral presentation).

European Society for Clinical Investigation,
Toulouse, April 1985 (abstract in European Journal
of Clinical Investigation (1985) 15: A52).

5. Could feverfew have anti-thrombotic potential? (poster presentation)

11th International Congress on Haemostasis and
Thrombosis, San Diego, July 1985.

Publications

1. Extracts of feverfew inhibit granule secretion in blood platelets and polymorphonuclear leucocytes.

Lancet (1985) (i) 1071-1074.

2. Neutrophils are involved in the increased vascular permeability produced by activated complement in man.

British Journal of Haematology (in press).

ABBREVIATIONS USED THROUGHOUT

| | |
|--------------------|---|
| ARDS: | Adult Respiratory Distress Syndrome |
| BSA: | Bovine Serum Albumin |
| CB: | Cytochalasin B |
| (N)CBP | (Neutrophil) Cobalamin Binding Protein |
| CGD: | Chronic Granulomatous Disease |
| CGL: | Chronic Granulocytic Leukaemia |
| CH50: | Total Haemolytic Complement |
| DMSO: | Dimethyl Sulphoxide |
| EACA: | Epsilon Amino Caproic Acid |
| EDTA: | Ethylene Diamine Tetraacetic Acid |
| EGTA: | Ethylene-bis (oxy-ethylene nitrile) Tetraacetic acid |
| FFE: | Feverfew Extract |
| FMPLP: | Formyl-Methionyl-Leucyl-Phenylalanine |
| 5 HETE: | 5 Hydroxy Eicosa Tetraenoic Acid |
| IEP: | Immunoelectrophoresis |
| LMWfr: | Low Molecular weight fraction |
| LTB ₄ : | Leukotriene B ₄ |
| NaAA: | Sodium Salt of Arachidonic Acid |
| NSAI: | Non-Steroidal Anti-inflammatory agent |
| PBS: | Phosphate Buffered Saline |
| PBS-A: | Phosphate Buffered Saline containing albumin, calcium and magnesium (see appendix 2). |
| PGE ₂ : | Prostaglandin E ₂ |
| PKC: | Protein Kinase C |
| PMA: | Phorbol Myristate Acetate |
| PMN: | Polymorphonuclear Leucocyte |

TLC: Thin Layer Chromatography

TXA₂: Thromboxane A₂

ZAS: Zymosan Activated Serum

EACA-ZAS Zymosan Activated Serum containing epsilon amino
caproic acid

GENERAL INTRODUCTION

Although the human polymorphonuclear leucocyte (PMN) has been considered primarily as a phagocytic cell, increased attention has recently focussed on its role as a secretory cell with involvement of its extracellular products in the processes of inflammation and immunity. PMN secretory events can occur independently of phagocytosis in response to soluble stimuli such as activated complement components and bacterial peptides. Products released include hydrolytic enzymes, inflammatory derivatives of arachidonic acid and membrane phospholipids, toxic oxygen radicals and binding proteins for iron and cobalamins such as vitamin B₁₂.

Secretory activities of PMN's are being increasingly implicated in the microvascular changes and tissue damage seen in a number of diseases associated with either complement activation and/or sepsis, such as the adult respiratory distress syndrome (ARDS) and rheumatoid arthritis.

PMN activation also occurs during elective surgery, with loss of secondary granules within hours¹, and a transient defect in cell killing associated with loss of primary granule myeloperoxidase². Investigation of the possible source of a stimulus to degranulation focussed on the complement system, although secondary granule secretion can also occur in serum without complement activation³. In view of the possible role of complement-mediated neutrophil secretion in the production of inflammation, this study has attempted to answer the following questions:-

1. Which complement components are the main secretory stimuli

for PMN's?

2. What are the other factors controlling PMN secretion?
3. Does PMN activation by complement lead to tissue inflammation, and if so, what are the mechanisms?
4. Can these tissue damaging processes be inhibited?

The experimental work falls into three sections. The first section examines secondary granule secretion from PMN's *in vitro*, both in the presence of activated complement and by other stimuli (Chapters 2 and 3). In the second section, an *in vivo* model of inflammation is developed, namely intradermal injection of activated complement and measurement of the resulting flare and wheal reaction. Identification of active complement components and the role of PMN activation in the response are then investigated (Chapter 4), followed by examination of the possible mechanisms of PMN involvement and ways of inhibiting the *in vivo* response (Chapter 5). Finally, in further *in vitro* studies, potential inhibitors of PMN secretion are examined (Chapters 6 and 7). The significance of the findings are discussed (Chapter 8).

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

SECTION 1. THE POLYMORPHONUCLEAR LEUCOCYTE : STRUCTURE, FUNCTIONS AND ACTIVATION PROCESSES.

A. Introduction

White blood cells or leucocytes are broadly divided on the basis of nuclear appearance into two types, mononuclear forms (monocytes and lymphocytes) and polymorphonuclear forms. The latter are further subdivided according to the staining characteristics of their cytoplasmic granules into eosinophils, basophils and neutrophils. Neutrophil polymorphs, otherwise known as neutrophil granulocytes, or polymorphonuclear leucocytes (PMN's, although strictly this term should include eosinophils and basophils also), are the most numerous white cell in the peripheral blood, numbers ranging from $1.8 - 7.3 \times 10^9/l^4$, and are 8-10 times more numerous than either eosinophils or basophils. The main function of the PMN is recognition, ingestion and killing of micro-organisms, and removal and repair of dead or damaged tissue. It thus has a primarily restorative function, though as will be discussed later in this chapter, PMN recruitment and activation may under certain circumstances lead to unwanted tissue injury.

B. PMN production and kinetics.

The lifespan of the PMN takes place sequentially in three body compartments, the marrow, the circulation and the tissues.

(i) Marrow

Generation of vast numbers of PMN's occurs in the bone marrow from stem cells which undergo a number of cell divisions to produce more differentiated forms (Table 1).

Table 1 (from Boggs 1975)⁵

Scheme of PMN maturation and storage in human bone marrow

| <u>Cell type</u> | <u>Average no</u> <u>divisions for</u> <u>each cell type</u> | <u>Cell output</u> <u>per stem</u> <u>cell</u> | <u>Approx</u> <u>transit time</u> <u>(days)</u> |
|------------------|--|--|---|
| Stem cell | ? infinite | 1 | Mitotic ↑ 5 ↓ |
| myeloblast | 1 | 2 | |
| promyelocyte | 1 | 4 | |
| myelocyte | 2 | 16 | Maturation ↑ 10-15 ↓ |
| metamyelocyte | - | 16 | |
| band | - | 16 | |
| segmented | - | 16 | Storage ↑ 2-5 ↓ |

The earliest stem cells in the maturation process are probably pluripotent i.e. they can produce progeny of any blood cell line⁶. Stem cells committed to myeloid or granulocyte differentiation then give rise to myeloblasts, and further cell divisions and maturation steps proceed through the promyelocyte and myelocyte stages till the non dividing

metamyelocyte form is reached. There is thereafter no further increase in numbers, but changes in the nucleus continue, giving rise firstly to the band form, and finally to the mature segmented PMN.

It can be seen from Table 1 that the total time spent by a PMN in the marrow is about 17-25 days⁵ of which 2-5 days is storage time once maturation is completed. This storage pool is enormous, consisting of between 7 and 13 times as many PMN's as in the peripheral blood⁵ and is of great importance in providing an immediate response to infection. The storage pool can be mobilised by endogenous or exogenous glucocorticoids⁷ or by the complement component C_3e ⁸. Segmented PMN's are released preferentially to band forms, although the latter may appear if demand is excessive. The presence of more immature forms such as myelocytes in the blood may be 'physiological' as in recovery from hypoplasia, but may also indicate an abnormal process in the marrow such as fibrosis or tumour invasion.

(ii) Circulation

Once in the peripheral blood, PMN's move between two pools of approximately equal size⁵, the circulating pool and the marginated pool which are cells adherent to blood vessel endothelium and therefore not included in peripheral blood PMN counts. Movement between these two pools is rapid, as shown by studies using labelled cells⁹, and the marginated pool can be rapidly mobilised by exercise, glucocorticoids or catecholamines¹⁰. Movement of cells from marrow to blood and from blood to tissues is presumed to take place via the marginated pool (Fig 1). Margination appears to take place

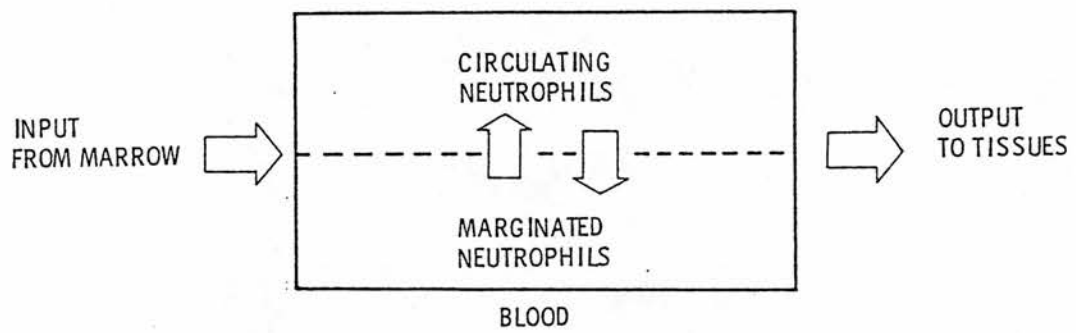


Fig 1. Movement of neutrophils between different body pools.

widely, with particular sites being lung, and to a lesser degree, liver and spleen⁹. The time spent by PMN's in the peripheral circulation, whether in margined or circulating pools, appears to be only a few hours, with a half life of labelled cells in man being only 6-7 hours¹¹. Since loss of labelled cells from the blood is exponential, it can be calculated that the average PMN spends about 10 hours in the bloodstream, and that the mass of blood PMN's turns over about two and a half times daily⁵. The exponential loss of labelled cells also suggests that loss to tissues is random, with no preferential loss of older cells, although there is also some evidence that PMN's may become senescent¹². Labelling studies also confirm that while PMN's move from marrow to blood and blood to tissues, there is little or no migration in the opposite direction¹¹.

(iii) Tissues

The general response of PMN's to tissue damage or infection begins with adhesion to endothelium followed by migration between endothelial cells (diapedesis) through into the tissue¹³. PMN's enter tissues even in health and spend 1-2 days there, where they undergo damage and death, being in turn phagocytosed by macrophages. PMN's in tissues do not constitute a mobile reserve of cells, so local tissue demands for increased numbers of PMN's can be met only by increased migration from the circulation¹⁴. Clearly, reduced PMN numbers in the peripheral blood will result in fewer available cells to enter damaged or infected tissue. If, however, neutrophilia is induced, no increase in migration is seen, numbers entering an infected site being proportional solely to

the intensity of the stimulus¹⁵.

C. The mature PMN : structure and functions

(i) Structure

The mature PMN (Fig 2) has a diameter of 12-14 μ ¹⁶ and a characteristic multilobed nucleus. The cytoplasm contains glycogen particles, important as an energy source, microfilaments and microtubules essential to the cell's mobility, and also important in phagocytosis and secretion, a few mitochondria and a small Golgi apparatus. The most striking feature of the cytoplasm, however, is the granules, which are of two main types with different constituents (Table 2).

Table 2

Contents of primary and secondary granules of human PMN's

(after Spitznagel 1974)

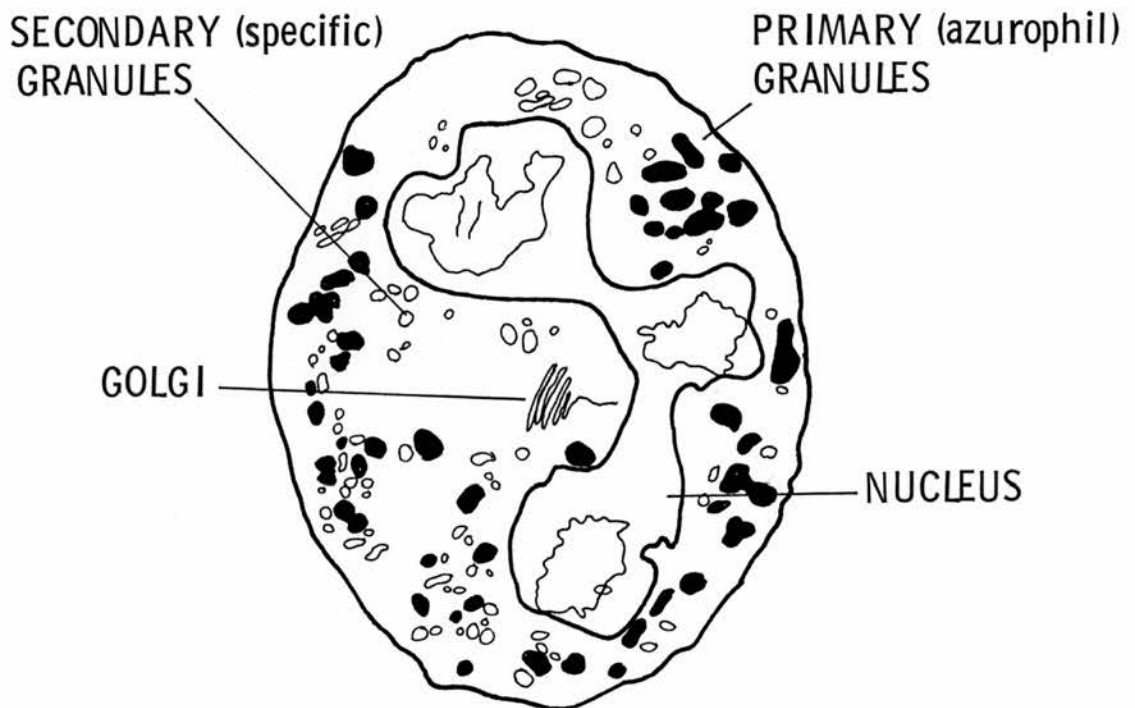
| <u>Primary</u> | <u>Secondary</u> |
|-------------------|---------------------------|
| Myeloperoxidase | Cobalamin binding protein |
| B glucuronidase | lactoferrin |
| neutral proteases | lysozyme (50%) |
| elastase | collagenase |
| cationic proteins | C ₅ activator |
| lysozyme (50%) | folate binding protein |

Alkaline phosphatase appears to exist in a completely separate population of granules.

The primary granules (also known as azurophilic, since they take up 'sky-blue' dyes, although their final stained colour is reddish purple) appear at the promyelocyte stage of cell development from the concave side of the Golgi apparatus¹⁸.

Their numbers are thereafter diluted by cell division. They

Fig 2. THE HUMAN POLYMORPHONUCLEAR LEUCOCYTE



contain mainly degradative enzymes and their function is to kill and break down material contained within the phagocytic vacuole or phagosome, with which they fuse¹⁹ (see (vii) below). The secondary or specific granules appear at the myelocyte stage of development and outnumber the primary granules by 2:1¹⁸. Although they contain the antibacterial protein lysozyme, the functions of their main constituents lactoferrin and cobalamin binding protein are not fully known. Since behaviour of the secondary granules constitutes a large part of this study, they are discussed more fully in part D of this section.

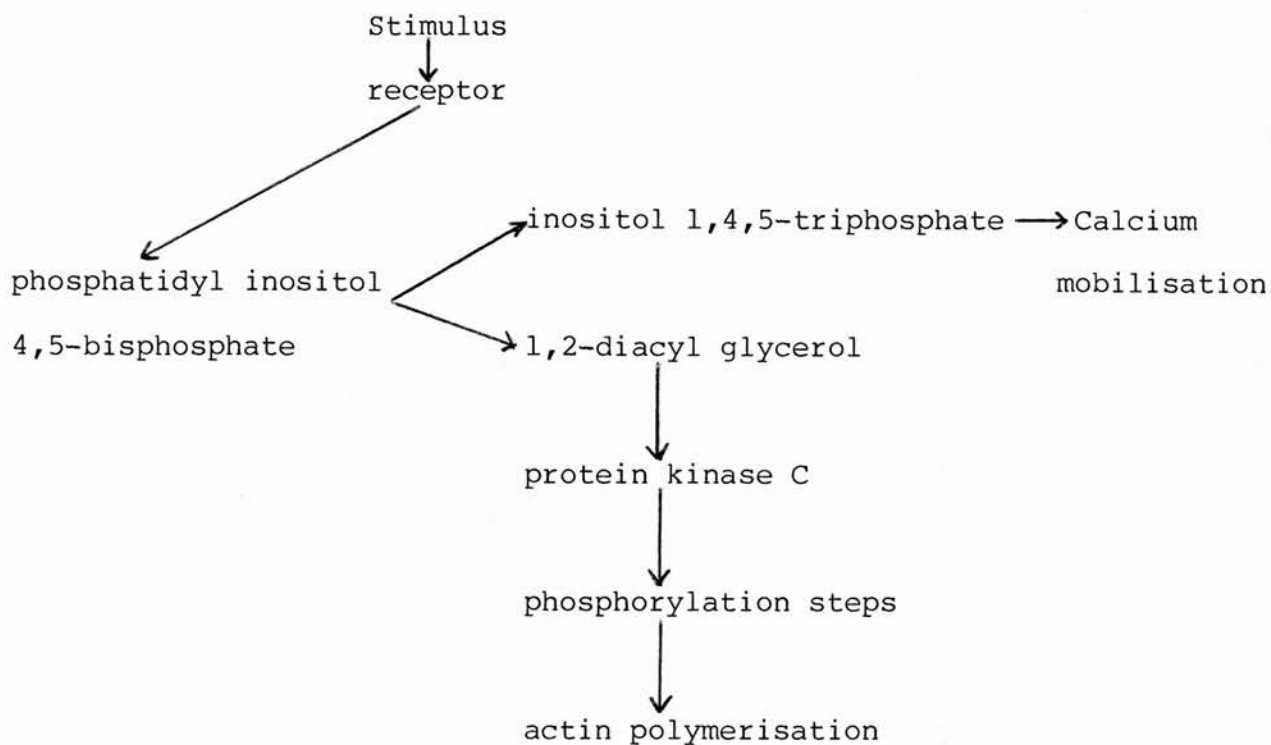
(ii) Responses to phagocytosable and soluble stimuli.

The mature PMN when stimulated is capable of a variety of responses, including chemotaxis, aggregation, degranulation, phagocytosis and killing, a respiratory burst, and production of biologically active mediators such as prostaglandins and leukotrienes. Which of these responses it displays in a given situation depends on whether the stimulus is particulate and therefore phagocytosable, bound to a surface too large to ingest, or soluble. Examples of soluble stimuli include the complement components C_{5a} and C_{5a} des Arg, discussed more fully in section 2 of this chapter, for which PMN's have receptors on their surface²⁰. Peptides from bacteria, which are generally formylated, also attach to surface receptors. An example is the tripeptide produced by *Escherichia coli*, formyl-methionyl-leucyl-phenylalanine²¹ (FMLP). Both C_{5a} and FMLP are used as PMN stimuli in this study. They can promote chemotaxis, granule release and a respiratory burst in the absence of any phagocytosable

stimulus^{21, 22}. PMN responses also differ according to whether the cells are in suspension, or whether they are attached to a solid surface²³.

(iii) Stimulus-response coupling.

Occupation of surface receptors for stimuli such as C_{5a} and FMLP results in a rapid reduction in membrane negative surface charge²⁴, and a rise in free cytoplasmic calcium, due at least partly to calcium loss from the cell membrane²⁵. Recent evidence suggests that biochemical events linking membrane receptor occupation with responses such as chemotaxis and degranulation may also involve breakdown of membrane phosphatidyl inositol 4,5-bisphosphate to form two intracellular messengers, 1, 2-diacyl glycerol and inositol 1, 4, 5-triphosphate²⁶. The former activates the membrane enzyme protein kinase C, which via a series of phosphorylation steps, leads to polymerisation of the actin and myosin-containing microfilaments responsible for chemotaxis, phagocytosis and granule release. Inositol 1, 4, 5-triphosphate, on the other hand, mobilises calcium from an intracellular store, possibly in the endoplasmic reticulum²⁷. Thus the rise in intracytoplasmic calcium may be preceded by these lipid changes, rather than being their cause. The events may be summarised thus:



(iv) Chemotaxis.

This complex process by which PMN's move on a surface along a chemical gradient, firstly involves detection by the cell via surface receptor occupation, of a directional difference in chemotactic factor concentration across its dimensions²⁸. The cell changes from round to wedge shaped, spreads out and finally moves in an amoeboid fashion. These processes appear to involve both actin and myosin-containing microfilaments, for locomotion, and also the microtubule system, for stability and orientation²⁹. Migration of cells exposed to attractants is accompanied by other receptor-mediated events, including a respiratory burst and degranulation. Secondary granule release involves fusion of the granule membrane with the plasma membrane with which it is identical and interchangeable. This may be important in renewing surface chemotactic factor receptors, which, with their bound ligands, are constantly lost from the cell surface

by internalisation²⁹. Degranulation thus provides the free receptors necessary for PMN migration.

The nature of the substratum on which the cell moves is also an important determinant of direction of migration. Cells will tend to follow grooves or dips in the surface, even if their orientation conflicts with the chemotactic gradient³⁰.

(v) Adhesion and recognition.

One process in the final maturation of the PMN is the development of surface receptors for two important immunological aids to phagocytosis namely C_{3b} , derived from the third component of complement, and the F_c end of the immunoglobulins IgG_1 and IgG_3 ³¹. Bacteria and viruses become coated with IgG antibody which then fixes complement to the bacterial surface. This process, known as opsonisation, greatly facilitates phagocytosis, for IgG opsonisation increases both affinity of the coated particle for the PMN receptor site as well as increasing the maximal rate of phagocytosis³². C_{3b} does not increase receptor affinity but does maximise the phagocytic rate.

(vi) Phagocytosis.

Phagocytosis takes place by the PMN forming two cytoplasmic outflowings (filopodia) round the particle. These then fuse, and the particle, along with some fluid and the relevant portion of plasma membrane becomes internalised forming the phagocytic vacuole or phagosome³¹. The phagocytic process, like chemotaxis, probably involves both microfilaments and microtubules, and divalent cations are required³³. Energy can be derived from anaerobic metabolism

of cytoplasmic glycogen³⁴.

(vii) Intracellular killing and degradation.

Microbicidal mechanisms are of two types, oxygen-dependent and oxygen-independent.

a. Oxygen-dependent. Phagocytosis is accompanied by an increase in oxygen consumption, which is converted by a cell membrane NAD(P)H oxidase (described more fully in the next section) to hydrogen peroxide (H_2O_2) and other reactive oxygen metabolites. Since the oxidase is incorporated, as part of the membrane, into the phagocytic vacuole, a high concentration of H_2O_2 can be generated within the vacuole³³. Although H_2O_2 is bactericidal alone, this activity is greatly enhanced by the presence of the enzyme myeloperoxidase, which becomes incorporated into the phagosome when it fuses with primary granules. An oxidisable cofactor such as halide ions is also required³³. Low levels of myeloperoxidase may underly the defect in killing found in pregnancy³⁵ and following surgery². Oxidative microbial damage may also occur independently of myeloperoxidase, by the reactive unstable products of oxygen reduction, namely the superoxide anion (O_2^-), singlet oxygen (O°) and hydroxyl radical ($OH\cdot$) as well as hydrogen peroxide itself³³ (see (viii) below).

b. Oxygen-independent. At least four systems are recognised:-

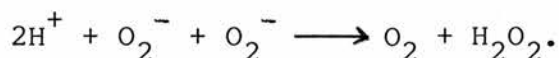
1. The low pH in the phagocytic vacuole, which may be due to accumulation of both lactic acid from glycolysis³⁶ and carbonic anhydrase-mediated carbonic acid production³⁷.
2. Lysozyme, which enters the phagocytic vacuole from the primary granules³⁸.

3. Cationic proteins, which are also found in the primary granules, and which can bind to and damage bacteria³⁹.

4. Lactoferrin, a secondary granule component. In its unsaturated form, lactoferrin can chelate the iron required for growth of certain bacteria³³. As discussed later in this section, its main site of action may be outside the cell.

(viii) The PMN oxidase and its deficiency.

As stated above, an increase in oxygen consumption or respiratory burst accompanies phagocytosis in human PMN's. As it is not inhibited by cyanide³³ and because PMN's contain few mitochondria, this appears to take place almost exclusively via the membrane NAD(P)H oxidase⁴⁰. As well as being activated during phagocytosis, the oxidase can be stimulated by soluble factors such as the complement component C_{5a}, particularly in the presence of serum¹⁴, and the bacterial peptide FMLP⁴². Under these circumstances, measurable amounts of superoxide anion (O₂⁻) appear outside the cell. Hydrogen peroxide can also be detected, generated probably from the dismutation of superoxide, thus:-



It is highly likely that other reactive oxygen species are also generated, and from their destructive effects on bacteria, it could be predicted that the extracellular appearance of such reactive moieties could cause unwanted damage to normal tissue. This is believed to be the case in certain disease states (see sections 3 and 4 of this chapter).

Deficiency of the PMN oxidase is seen in the inherited condition chronic granulomatous disease, in which impaired function of the microbicidal myeloperoxidase-halide-hydrogen

peroxide system leads to chronic sepsis from early in life with both Gram positive and Gram negative bacteria, which are ingested but not killed⁴⁰. There is no respiratory burst during phagocytosis, which can be detected by failure of reduction of nitroblue tetrazolium³³, or by direct measurement of superoxide or hydrogen peroxide.

D. PMN secondary granules and their extra-cellular functions.

(i) Contents (see Table 2)

The secondary or specific granules of PMN's contain about half the cell's lysozyme, and practically all the intracellular lactoferrin¹⁷ and cobalamin binding protein (CBP)⁴³, also referred to as vitamin B₁₂ binding protein although it binds other cobalamins as well as vitamin B₁₂ or cyanocobalamin.

(ii) Responses to stimuli

Secondary granules respond quite differently from primary granules during either phagocytosis or exposure to soluble stimuli. Phagocytosis produces a degree of secondary granule fusion with the phagosome¹⁹, but most of the secondary granule components CBP⁴⁴ and lactoferrin⁴⁵ appear extra-cellularly. In contrast, very little primary granule content appears extracellularly during phagocytosis⁴⁴. A similar disparity is seen when considering extracellular granule release in response to a variety of soluble stimuli. For some stimuli e.g. the phorbol ester phorbol myristate acetate, significant secondary granule release occurs with absolutely no primary granule discharge at all⁴⁶. For other stimuli such as C_{5a} or FMLP, secondary granule release takes

place both more rapidly and to a much greater extent than does primary granule release⁴⁷. This sequential release of granule contents would support the hypothesis that the two granule types are controlled by different mechanisms.

(iii) The use of cytochalasin B in degranulation studies

Cytochalasin B (CB) is a fungal metabolite which prevents the polymerisation of cytoskeletal actin necessary for phagocytosis⁴⁸. When presented to the cell along with a phagocytic stimulus, CB thus blocks phagocytosis and the primary granules discharge themselves not into the phagosome, but into the extracellular environment⁴⁹. Addition of CB to PMN's along with a soluble stimulus, however, greatly speeds up and magnifies both primary and secondary granule release⁴⁹, with secondary granule release beginning in seconds and reaching a plateau in 2 or 3 minutes, as opposed to the 45 or 60 minutes needed in the absence of CB. Despite these major effects, CB causes little or no degranulation when added in the absence of a stimulus.

CB has become widely used in experimental studies of control of degranulation and investigation of the actions of degranulation inhibitors. It may be argued that its use is unphysiological and renders interpretation of results open to question. On the other hand, it has been pointed out that the study of PMN's in suspension is in itself highly unphysiological, as responsive PMN's *in vivo* are almost invariably adherent to a surface²³, be it vascular endothelium, collagen or fibrin. The addition of CB to *in vitro* studies, it has therefore been argued, actually restores conditions to a more natural state by substituting

for an adherent surface, and allowing the cells to behave as they would *in vivo*²³. CB has been used in this study as an enhancer of stimulus-induced secondary granule release, although many comparable experiments have been performed without it. The role of surfaces in the PMN response to *in vivo* inflammation is further discussed in section 4 of this chapter.

(iv) The extracellular functions of PMN secondary granule components.

a. Lysozyme. Its actions on bacteria outside the cell are probably similar to those seen when it enters the phagosome. In addition, it appears to inhibit PMN chemotaxis⁵⁰, thus localising PMN's to inflammatory sites. Lysozyme also appears to act as a negative feedback of PMN activation, for there is some evidence that it suppresses superoxide generation⁵⁰.

b. Cobalamin binding protein (CBP). This occurs with lactoferrin in many body fluids, as well as in PMN secondary granules, where it is in a completely unsaturated form. There are several different cobalamin binding proteins (transcobalamins) in the body, with different binding affinities for the various cobalamins and corrinoids, of which cobalamins are a subgroup. The CBP in neutrophils is of the R binder type i.e. it particularly binds cobalamin analogues of vitamin B₁₂ (cyanocobalamin) rather than the vitamin itself⁵¹. Its function is not known, although it may have a role in removing cobalamin analogues produced by bacteria.

There may also be a binding protein for folate in PMN secondary granules, (Sheppard K, unpublished observations) but

as yet this is poorly characterised.

c. Lactoferrin. This iron binding protein is present in PMN's in a completely unsaturated form, so that it readily binds any free iron present in the surroundings. Since iron is a growth factor for certain bacteria, this chelating action may be protective³³. Lactoferrin also promotes PMN adhesion both to other PMN's and to endothelium, a property which may amplify the inflammatory response⁵². Lactoferrin may additionally have a role in bacterial killing, for in the rare inherited condition of specific granule deficiency, moderate impairment of bactericidal activity is seen⁵³. While the exact basis for this is not known, it may be indirectly due to lack of lactoferrin-bound iron, which can promote the production from superoxide of the highly toxic hydroxyl radical⁵⁴. A further important role for lactoferrin appears to be in the control of PMN production (granulopoiesis). The growth of myeloid or granulocytic colonies in culture depends on the presence of one or more colony stimulating factors (CSF) produced via a series of complex interactions between monocytes and lymphocytes⁵⁵. Cell-free supernatants from activated PMN's can exert an inhibitory effect on CSF production⁵⁶, and there is now evidence that lactoferrin may play a major role in this control process⁵⁷.

It can thus be seen that extracellular discharge of PMN secondary granules plays an important role in the inflammatory response, as do also biologically active lipid mediators generated on cell stimulation. A fuller discussion of the effects of these and of PMN products generally in inflammation and tissue damage follows in section III of this chapter.

SECTION II. THE COMPLEMENT SYSTEM AND ITS EFFECTS ON NEUTROPHILS

A. Functions and activation pathways

The human complement system consists of a series of nine plasma proteins (C1-9) which circulate in an inactive state, and which, when activated, undergo a cascade of cleavage reactions resulting in the generation of biologically active peptides, and of complexes which can damage the membranes of bacteria, viruses and foreign cells. In addition, attachment of complement proteins, particularly C_{3b} , to foreign material assists in its adhesion to and phagocytosis by PMN's and macrophages. Thus the system exerts a primarily protective function, although endogenous tissue damage may also result from complement activation. There are two pathways by which the system may become activated, each with its own control proteins. The classical pathway is primarily activated by antigen-antibody complexes, while the alternative pathway is triggered largely by foreign material and cell surfaces⁵⁸ (Table 3). The later steps in each pathway are identical. The classical and alternative pathways (Fig 3) differ in their requirement for divalent cations, the former being calcium dependent, while the latter requires magnesium.

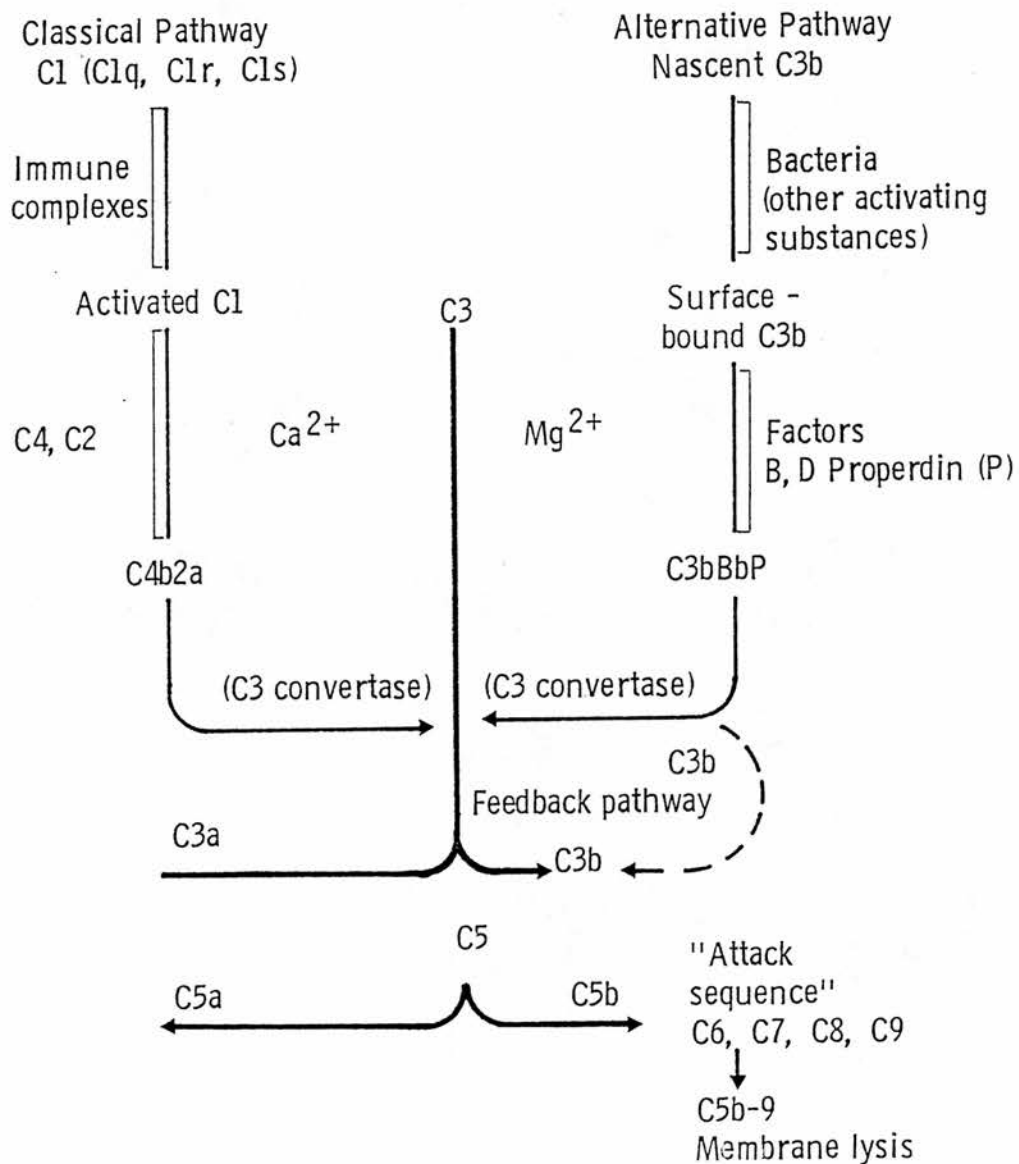


Fig 3. The classical and alternative complement pathways.

Table 3

Factors which activate classical and alternative
complement pathways

| <u>Classical</u> | <u>Alternative</u> |
|---|---|
| Antigen-antibody complexes | Complex polysaccharides (e.g. inulin) |
| Antigen may be soluble or a surface component | Bacterial lipopolysaccharides (endotoxins) |
| Antibodies must be either IgM, IgG, IgG ₂ IgG ₃ | Yeast cell walls (zymosan) |
| | Cell surfaces of certain bacteria and fungi |
| | Certain immune complexes e.g. containing IgA or IgD |
| | Dialyser membrane cellophane |

Modified from Goldstein 1980⁵⁸.

B. Anaphylotoxins

It is crucial to the understanding of the complement system to appreciate that many active conversion products of complement proteins will remain attached to the target cell e.g. C_{3b}, C_{5b}, and will fix to that site later components in the pathway. These solid phase components contrast with a number of smaller proteins, e.g. C_{3a}, C_{5a} and C_{4a}, known collectively as anaphylotoxins, which can diffuse through tissue fluids and set up concentration gradients with effects on motile cells such as PMN's and monocytes. In addition, such proteins can interact with fixed cells such as mast cells and tissue macrophages, triggering secretion and thereby amplifying the tissue response to infection. The term anaphylotoxin refers to the ability of C_{3a} and C_{5a} to trigger

severe anaphylactic reactions in animals and man, with collapse, hypotension, bronchospasm and death. This property appears to be mainly due to mast cell activation, with release of histamine and other mediators, leading to contraction of bronchial and vascular smooth muscle⁵⁹. Indeed contraction of guinea pig ileum is one traditional biological assay for these mediators. A further property of C_{3a} and C_{5a} is the production of increased microvascular permeability in the skin⁵⁹. All properties of C_{3a} and C_{5a} are displayed down to nanomolar concentrations⁶⁰. Not surprisingly, these powerful molecules have a half-life of only a few minutes *in vivo*, being acted upon by a plasma enzyme carboxypeptidase B, which removes their carboxy terminal amino acid arginine generating C_{3a} des Arg and C_{5a} des Arg respectively⁶¹. Although these stable derivatives are 100-1000 times less active towards mast cells than C_{3a} or C_{5a} ⁶², they remain active in their effects on PMN's and macrophages, and C_{5a} des Arg remains a potent producer of increased vascular permeability⁶⁰ (see Table 4). The anaphylotoxic properties of C_{4a} are 2-4 orders of magnitude less than those of C_{3a} or C_{5a} ⁶⁴.

Table 4

Biological activities of C_{3a} , C_{5a} and their des Arg derivatives

| | C_{3a} | C_{3a} des Arg | C_{5a} | C_{5a} des Arg |
|---------------------------------|----------|---------------------|----------|---------------------|
| Contraction of smooth muscle | + | - | + | - |
| Mast cell degranulation | + | - | + | - |
| Increased vascular permeability | + | ? | + | + |
| PMN chemotaxis and activation | - | - | + | + |

* may require 'helper factor' or 'cochemotaxin' from plasma⁶³

C. Interactions between C_3 and C_5 fragments and PMN's.

This study is particularly concerned with the effects of C_{3a} and C_{5a} and their des Arg derivatives on PMN activation and degranulation *in vitro*, and on the micro-circulation and permeability control *in vivo*. One of the technical difficulties in describing the actions of C_3 and C_5 cleavage products is that native C_3 and C_5 are structurally very similar, as are C_{3a} and C_{5a} , although human C_{5a} has a small carbohydrate side chain⁶⁵. The methodological limitations to the separation of these molecules were not overcome till 8 or 10 years ago, so that in discussing the biological actions of C_{3a} and C_{5a} , the literature prior to the mid 1970's referred to both of them as chemotactic factors for PMN's. More recent work on the

effects of C_{3a} and C_{5a} on PMN's however has cast doubt on whether C_{3a} or its des Arg derivative have any effects on PMN's at all. A careful study of chemotaxis showed that neither C_{3a} nor C_{3a} des Arg could activate PMN's, and proposed that earlier results suggesting that they could were due to contamination of C_{3a} preparations by small amounts of potent C_{5a} ⁶⁶. This point will be further examined in this study.

C_{5a} and C_{5a} des Arg are both capable of activating PMN's through the same receptor although the des Arg form has reduced binding affinity²⁰, and its ED_{50} for PMN effects is 10-20 times higher²². (See Table 5)

Table 5

Concentrations of human C_{5a} and C_{5a} des Arg
which produce various responses from polymorphonuclear
leucocytes

| | C _{5a} | C _{5a} des Arg |
|---|-----------------------------|-------------------------|
| | ED ₅₀ values (M) | |
| Chemotaxis | | |
| Boyden chamber method | 0.9x10 ⁻⁹ | 2.0x10 ⁻⁸ |
| Agarose method | 1.0x10 ⁻⁹ | 6.3x10 ⁻⁸ |
| Skin window technique (rabbit PMN's) | 1.7x10 ⁻⁷ | 3.3x10 ⁻⁷ |
| PMN receptor affinity | 3-7x10 ⁻⁹ | N.D. |
| B glucuronidase release | 1-5x10 ⁻⁹ | 1-8x10 ⁻⁷ |
| O ₂ - generation | 3-6x10 ⁻⁸ | 1-3x10 ⁻⁶ |

N.D. = Not determined

Combined data from Hugli 1979⁶⁰ and Webster et al 1980²².

The estimated concentration of either C_{5a} or C_{5a} des Arg which could be generated in maximally activated plasma is $3-8.5 \times 10^{-7} \text{ M}$ ⁶⁰.

Since C_{5a} and C_{5a} des Arg are always produced together and because their effects on PMN's are qualitatively similar, they will henceforth be described collectively as C₅ fragments, unless one or other is specifically stated.

Although C₅ fragments are well known for their chemotactic properties towards PMN's, chemotaxis is but one response of the cell to such stimuli. If the cells are in suspension, aggregation follows within minutes, accompanied by

release of secondary granule components and generation of superoxide via the membrane oxidase²². More intense stimulation can also lead to primary granule discharge⁶⁷. Involvement of such mechanisms in the production of tissue damage in various disease states is discussed in the next section.

D. Inactivation and clearance of C_{5a} des Arg

Two mechanisms of inactivation of C_{5a} des Arg have been described. The first is a plasma inactivator of many different chemotactic factors first described in 1973⁶⁸, known as chemotactic factor inhibitor (CFI). Prolongation of the plasma half-life of C_{5a} des Arg injected intravenously into rabbits by protease inhibitors such as aprotinin may represent blockage of CFI⁶⁹. CFI, which may have more than one component⁷⁰, has been reported as being elevated in certain conditions such as sarcoidosis, Hodgkin's disease and cirrhosis⁷¹. The significance of this observation to the pathogenesis of these diseases remains unclear.

The second way by which C_{5a} des Arg is cleared from the circulation and tissues appears to be via PMN's themselves. Once C_{5a} des Arg has bound to PMN surface receptors, the C_{5a} des Arg-receptor complex becomes internalised, and the C_{5a} des Arg is no longer available⁷². The plasma half-life of labelled C_{5a} ⁷³ and C_{5a} des Arg^{69, 73} was prolonged in neutropenic rabbits, almost entirely due to reduced splenic uptake of the label. Accumulation in the lungs, however, was independent of circulating PMN's and was probably due to C_{5a} and C_{5a} des Arg adhering directly to the vascular endothelium⁷³. This may have bearing on the lung damage

believed to be produced by activated complement and PMN's in the adult respiratory distress syndrome or shock lung, described in section III of this chapter.

E. Detection of complement activation.

Although assay of non-activated (native) complement components such as C_3 and C_4 by radial immunodiffusion is now standard in immunological laboratories, such measurements give little information about the state of complement activation or turnover, since increased conversion and breakdown of C_3 and C_4 may be offset by increased synthesis. Such information can be gained only by assay of fluid phase activation products such as C_3 or C_5 fragments. At present, this is readily possible only for C_3 fragments. Methods available include 2 dimensional immunoelectrophoresis (measuring mainly C_{3c} and C_{3d}) and rocket electrophoresis for C_{3d} ⁷⁴. Such methods are not however sensitive enough to detect the tenfold lower concentrations of C_5 fragments produced even in maximally activated plasma⁶⁰, and for this reason, biological assays have till now been their main method of detection. The traditional anaphylotoxin assay employing contraction of guinea pig ileum has however two major drawbacks, as well as being time-consuming and technically complicated. Firstly, it does not differentiate between C_{3a} and C_{5a} , which both have anaphylotoxic properties, and secondly it does not detect the stable and potent PMN activator C_{5a} des Arg. Assays utilising the unique actions of C_5 fragments on PMN's have therefore been devised. These include PMN aggregation⁷⁵ and degranulation, which is investigated in this project. Finally, radioimmunoassay for C_{5a}/C_{5a} des Arg is becoming

available as a research tool. The relative advantages and disadvantages of these various assay systems are discussed more fully in chapter 2.

SECTION III. THE ROLE OF NEUTROPHIL ACTIVATION BY CHEMOTACTIC
COMPLEMENT FACTORS IN HUMAN DISEASE.

Clearly the most commonly occurring situation in which generation of C_5 fragments leads to PMN recruitment and activation is in local tissue infection, where demarcated self limiting tissue injury is seen, but which resolves without major problem.

In other circumstances, however, interaction between C_5 fragments and PMN's may have more serious consequences. Situations in which C_5 fragment generation and/or neutrophil activation are believed to be important in disease production are listed in Table 6.

Table 6

Situations in which complement and/or PMN activation may be
important in disease production in man.

1. Adult respiratory distress syndrome.
2. Haemodialysis and cardiopulmonary bypass.
3. Following surgery or burns.
4. Rheumatoid arthritis and gout.
5. Myocardial infarction.
6. Sepsis.

A. Adult Respiratory Distress Syndrome (ARDS - synonyms include shock lung and fat embolism syndrome).

This puzzling condition, carrying a 60-70% mortality⁷⁶, complicates a number of disorders including septicaemia, particularly with Gram negative organisms, multiple trauma,

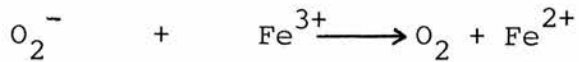
pancreatitis and thermal injury⁷⁷. The earliest clinical features are dyspnoea and hypoxia, and although the chest Xray may be initially unremarkable, patchy shadowing quickly develops across both lung fields. A protein-rich alveolar exudate develops so that the lungs become stiff, and artificial ventilation requires high inspiratory pressures. There is no specific therapy, beyond support and treatment of the antecedent condition, although some centres claim benefit from very high dose steroids i.e. in gram quantities⁷⁸. Although the exact pathogenesis of the condition is not fully known, evidence is accumulating from both animal and human studies that complement and/or PMN activation plays a part in the abnormal permeability of the pulmonary vasculature and the resulting lung damage. For example, the increase in pulmonary capillary permeability to labelled proteins was increased in sheep following experimental pancreatitis⁷⁹ and in guinea pigs following challenge with intravenous pneumococci⁸⁰. In either case, the permeability change was absent or reduced in neutropenic animals. Administration of pre-prepared C₅ fragments either intravenously^{80, 81} or directly into the lungs⁸² can also produce some of the changes of ARDS, with PMN adhesion to pulmonary endothelium, migration into alveolar spaces, and increased vascular permeability which is again preventable by neutropenia⁸⁰. Observations in human ARDS have highlighted the frequent development of transient neutropenia immediately prior to the appearance of clinical signs⁸³, thought to be due to PMN trapping in the lungs. PMN's entering the lungs may have already been 'primed', with increased basal superoxide production in pulmonary artery

PMN's⁸⁴, and increased basal enzyme release with impaired chemotaxis in peripheral blood PMN's⁷⁶. Neutrophil elastase has also been found in bronchial lavage fluid from ARDS patients⁸⁵.

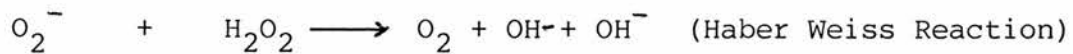
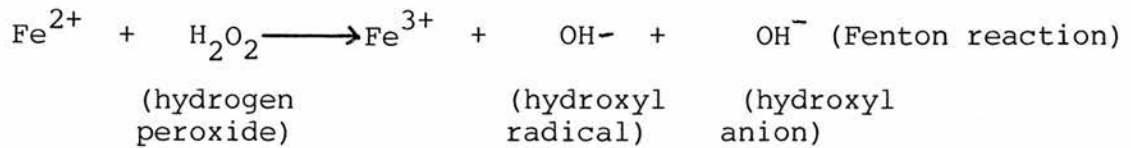
In a review of experimental complement-mediated ARDS, Henson points out that production of the full picture of ARDS may also require an additional insult such as hypoxia⁸⁶, and also stresses that although C₅ fragments undoubtedly cause PMN aggregation *in vitro*, they may trigger PMN adhesion to endothelium rather than to each other, when present in the circulation. Indeed the endothelium itself may be altered by C₅ fragments such that PMN/endothelial adhesion is enhanced. Other contributory factors may include pulmonary macrophages which can be acted upon by PMN granule glycoproteins⁸⁷, and which may compound the situation by the release of enzymes capable of generating further C₅ fragments from C₅⁸⁸, and by the synthesis of chemoattractant lipid products such as platelet activating factor and leukotrienes.

One metabolic feature of the PMN which may be important in the production of lung damage is the membrane oxidase which generates the superoxide anion and hydrogen peroxide from molecular oxygen. Lung damage produced in animals by the complement activator cobra venom factor could be prevented by either the hydroxyl radical scavenger dimethyl sulphoxide or the ionic iron scavengers desferrioxamine and apolactoferrin (but not iron saturated lactoferrin)⁸⁹. This has led to the hypothesis that generation of the highly reactive hydroxyl radical, a reaction catalysed by Fe³⁺, is an important step in the production of increased vascular permeability, perhaps via

peroxidation of membrane lipids. The reactions are:-



(superoxide
anion)



One proposed mechanism of ARDS production is therefore that C_5 fragments generated either in the circulation or in the lungs, increase adhesion between PMN's and pulmonary capillary endothelium either by altering either the PMN or endothelial cell membrane, or both. Migration of PMN's through into alveoli may then follow, with release of granule contents and toxic oxygen products, leading to increased vascular permeability and exudate production. Possible mechanisms for this will be discussed further in the next section. Some of the lungs' protective mechanisms such as α_1 antitrypsin may also be destroyed by oxidation⁸⁵. Other cell types, such as pulmonary macrophages and platelets, may also play a part in the production of some of the features of the condition, but complement and PMN activation appear to be central.

Several points of interest emerge from this hypothesis. Firstly, the role of Gram negative sepsis, and particularly of endotoxin, appears to be a major one⁹⁰. Haemorrhagic

shock alone, for example, does not appear to cause ARDS in man. As well as being a potent activator of the alternative complement pathway, endotoxin may alter pulmonary endothelium directly, rendering it 'sticky' and more liable to trap PMN's by preventing release of temporarily marginated cells.

Secondly, the possible therapeutic benefit of high dose steroids in ARDS may at least in part be explained on the basis of an inhibitory effect on PMN's, for high concentrations of hydrocortisone inhibit PMN degranulation *in vitro* in response to either C_{5a} or FMLP⁹¹. Although the millimolar concentrations required for inhibition of degranulation *in vitro* are much higher than those achieved by 'normal' steroid dosage *in vivo*, intravenous administration of the 30mg/kg of methyl prednisolone recommended for shock by the current British National Formulary (1985) means that these millimolar levels may be approached in plasma.

Thirdly, it is apparent that clinical diagnosis of ARDS, based on radiological, blood gas, and ventilatory criteria, can only be made once the condition is already well advanced pathologically. While prevention of PMN involvement may be possible with high dose steroids, little can be done once lung damage supervenes. If free C_5 fragments can be detected in the circulation of ARDS patients, and if, as is claimed, their detection is a better predictor of ARDS than the currently available C_3 fragment detection by immunoelectrophoresis⁹², then a reliable, robust detection system for C_5 fragments should prove a valuable tool in ARDS detection and management. This aim is further elaborated in chapter 2.

B. Extracorporeal circulation of blood : haemodialysis and cardiopulmonary bypass.

The observation that when a cellophane membrane was used for haemodialysis a transient neutropenia and monocytopenia was seen during the first hour of dialysis, led to the finding that dialysis cellophane can activate complement⁹³. Infusion of plasma leaving the dialyser into rabbits produced identical leucopenias, with PMN entrapment in pulmonary capillaries⁹³. Although dialysis neutropenia is now well documented it rarely gives rise to clinically significant pulmonary damage. Similar activation of C_3 has been seen in cardiopulmonary bypass^{94, 95}, with the nylon mesh liner of bubble oxygenators being a potent complement activator, as well as the oxygenation process itself⁹⁵. Although elevated levels of free C_{5a} in association with bypass have not been reported, the neutrophilia pertaining during bypass may be followed by a neutropenia immediately on reestablishment of cardiopulmonary circulation, suggesting pulmonary entrapment of C_{5a} -coated granulocytes⁹⁵. Whether this phenomenon is responsible for any of the pulmonary complications of cardiopulmonary bypass is as yet unclear.

C. Post-operatively and following burns.

The complement and neutrophil changes in ARDS may differ only in degree from the effects of much less severe trauma, such as elective surgery. Elective gastric surgery, for example, is frequently associated with rapid conversion of C_3 and a fall in C_3 and C_5 levels⁹⁶. A study of patients undergoing elective hysterectomy has also shown evidence of intravascular release of PMN secondary granules, with

intracellular levels of both lysozyme and NCBP falling to about 60% of their pre-operative levels within four hours of skin incision¹. The changes were not seen in patients undergoing minor procedures only, and could not be accounted for by anaesthesia, endogenous release of glucocorticoids or release of young cells from the marrow. Plasma levels of both NCBP and lysozyme both rose post operatively and were significantly elevated by the fifth post operative day. Similarly, loss of PMN lysozyme has been seen following burns⁹⁷, as have raised plasma levels of another secondary granule marker, lactoferrin⁹⁸. Interestingly, intravenous administration of the chemotactic peptide FMLP into rabbits has resulted in both transient neutropenia and raised plasma lactoferrin⁹⁹, confirming that stimulus-mediated lactoferrin release from PMN's can occur intravascularly. The clinical significance of such changes following surgery and burns is not clear, although in both circumstances there are also defects in neutrophil function^{2, 97}.

D. Rheumatoid Arthritis and Gout.

Although rheumatoid arthritis is characterised by the development of lymphocyte-derived rheumatoid factors, a prominent PMN infiltrate is seen in synovial fluid and in the synovium itself, where some of the tissue damage may be caused by PMN proteases¹⁰⁰ and oxygen products¹⁰¹. Evidence of classical complement pathway activation is also seen in synovial fluid with isolation of C₃ breakdown products, C_{5a} and the C₅₆₇ complex¹⁰⁰, and low levels of C₁, C₄ and C₂¹⁰². Such complement activation is not a feature of traumatic or degenerative joint conditions.

C_{5a} generation and neutrophil activation may also play a part in gout. Monosodium urate crystals can not only activate the alternative complement pathway¹⁰³, but may trigger PMN's directly. Indeed, joint damage due to intra-articular urate may depend on the presence of PMN's¹⁰⁰.

E. Myocardial Infarction.

There is evidence for plasma activation of the classical complement pathway in the first 72 hours following acute myocardial infarction in man¹⁰⁴. In experimental infarction in dogs, prior complement depletion using cobra venom factor significantly reduces both PMN infiltration and extent of myocardial necrosis¹⁰⁵. The zone at the edge of an area of established infarction usually receives an early influx of PMN's and may itself then become infarcted. Infusions of PMN-inhibiting anti-inflammatory drugs such as the cyclooxygenase inhibitor ibuprofen¹⁰⁶ and the combined cyclooxygenase/lipoxygenase inhibitor BW755C¹⁰⁷ both attenuate this PMN infiltrate and reduce infarct size significantly, with no effect on platelet infiltration. Moreover, induction of neutropenia prior to experimental infarction also results in smaller infarcts¹⁰⁷.

F. Sepsis.

In discussion of ARDS, myocardial infarction and gout, the basic hypothesis has been the same, i.e. that unwanted PMN activation by C_5 fragments or other complement components leads to the extracellular release from PMN's of tissue damaging compounds, and that inhibition of such PMN involvement might prevent some of the clinical manifestations.

Excessive exposure of PMN's to C_5 fragments or other soluble stimuli may have another effect however, which is to prevent the cell responding appropriately to an infective agent. As mentioned previously, both surgery and burns are conditions in which complement and PMN activation appear to occur in the absence of infection. Both are also associated with defects of PMN function, namely reduced intracellular killing postoperatively², and impaired chemotaxis with loss of granules following burns⁹⁷. It may therefore be that once the cell has been initially activated by C_{5a} and partially degranulated, a state of unresponsiveness ensues.

SECTION IV. IN VIVO MODELS OF NEUTROPHIL-MEDIATED TISSUE

DAMAGE

A. Introduction

Attempts to relate the *in vitro* responses of PMN's to the *in vivo* signs of inflammation have been in progress for over thirty years. The cardinal signs of inflammation, namely rubor (redness), calor (heat), tumor (swelling), and dolor (pain) are almost invariably accompanied by a marked PMN infiltrate, but the ways in which these relate to one another are not yet fully unravelled. The aspects of PMN-related inflammation with which this study is concerned are the vasodilatation responsible for both heat and redness, and the increase in vascular permeability which must precede the formation of tissue oedema and swelling. The work attempts to explore the ways in which these changes result from PMN-complement interactions. Most previous studies of this nature have been done, for obvious reasons, in animals, but since there is considerable interspecies variation in the properties of certain complement components, extrapolation from animal studies to man is not always valid. However, a review of some animal models will demonstrate how the problem has been approached.

B. Early descriptive studies of PMN-dependent tissue damage

In the late 1940's and 1950's, before immunoglobulin and complement components could be purified, a number of models were used to produce inflammatory tissue damage in the skin of animals. Two such models were the Arthus and Schwartzman phenomena, which were recognized as having many

features in common¹⁰⁸. The Arthus phenomenon is produced by giving an animal repeated intradermal injections of an antigen till it becomes sensitized i.e. produces an appropriate antibody response. Further injections of antigen at the same site then produce local necrosis, with a histological picture showing clumps of PMN's in vessels and migrating into the tissues¹⁰⁹. A slightly different technique, the Reversed Passive Arthus reaction (RPA), involves giving the antigen intravenously followed by intra-dermal injection of antibody. A similar histological picture results¹¹⁰. The Schwartzman phenomenon is produced by injecting bacterial endotoxin into the skin, followed 24 hours later by an intravenous injection of the same endotoxin, and was first described by Schwartzman in 1928¹¹¹. Two to six hours after the intra-dermal injection, a haemorrhagic skin reaction develops locally, with evidence of PMN accumulation and vascular injury histologically. Unlike the Arthus reaction, agglutinating or precipitating antibody is not involved¹¹². With present day knowledge, it is clear that the Arthus reaction involves the development of complement fixing antibodies. The exact mechanism of the Schwartzman reaction is less clear, but probably, as with both the Arthus and RPA reactions, includes complement activation. Generation of C_5 fragments would then result in PMN recruitment.

The technical limitations of the time did not prevent investigators making astute observations and speculations as to how the tissue damage of the Arthus and Schwartzman phenomena might be being produced. These included the

following:-

1. In 1948, Becker produced suppression of the Schwartzman reaction in the rabbit by benzol (the alternative name for benzene), irradiation and nitrogen mustard, provided they were given 2-3 days before the intravenous injection¹¹².

He concluded, however, that:

"suppression by these agents is exerted through their specific but common suppressive action on the reticulo-endothelial system, primarily the vascular endothelium. These endothelial cells being rendered anergic are not able to react to the active principles in a way that otherwise would be self-destructive"

We do not now think that these agents would significantly alter vascular endothelium, but were probably working via marrow suppression.

2. In 1951, Stetson and Good, also using rabbits, confirmed the suppressive effects of benzene and nitrogen mustard on the Schwartzman reaction, and found that this suppression correlated closely with the degree of neutropenia¹¹³. Lack of suppression was seen in animals whose marrow was protected from the effects of the nitrogen mustard.

3. Stetson also observed that the intravenous injection of antigen was accompanied by a transient neutropenia¹¹⁴ prior to the onset of skin necrosis, and that clumps of PMN's and platelets were seen plugging capillaries in the lungs and other organs. These observations bear close resemblance to changes seen in experimental models of ARDS.

Following these studies, Stetson drew attention to the similarities in development of Arthus and Schwartzman lesions, viz:-



- (i) perivascular PMN accumulation in affected skin area
- (ii) increased aerobic glycolysis in injected area
(probably due to the large numbers of PMN's)
- (iii) production of leucocyte and platelet blockage of
capillaries locally and elsewhere
- (iv) transient systemic neutropenia
- (v) haemorrhage from affected vessels¹⁰⁸.

4. Observations on the Reversed Passive Arthus reaction in the rabbit were made by Humphrey in 1955¹¹⁵, who again demonstrated suppression of the reaction by exposure of marrow to nitrogen mustard. Partial restoration of responses could be obtained by local injection of harvested exudate PMN's from other animals. These PMN's would have been already activated and partly granule depleted by the initial harvesting procedure, so lack of complete restoration of responses is not surprising. Humphrey also formally demonstrated an increase in capillary permeability and showed marked inhibition of responses by cortisone or ACTH and partial reduction by sodium salicylate, though not by antihistamines¹¹⁵. He attributed these findings to an effect on endothelium, but inhibition of PMN activation is an alternative possibility⁹¹.

5. In 1965, Kniker and Cochrane induced yet another type of antigen-antibody mediated tissue reaction in rabbits, namely serum sickness¹¹⁶. In this model, antigen-antibody complexes, formed in antigen excess and hence soluble, are deposited in the vessel walls of many organs, including kidneys, joints and heart. Lesions in coronary arteries show endothelial proliferation and damage to the internal elastic

lamina, with sub-endothelial fibrinoid and damage to the media. In animals rendered neutropenic, although immune complexes could still be demonstrated in vessels by immunofluorescence, coronary artery damage was much reduced, and damage to the internal elastic lamina was absent.

C. Quantitative studies using purified chemotactic factors.

Studies which have attempted to quantify the vascular permeability changes and other skin changes produced by purified complement components and other chemotactic stimuli, have been notably performed by two groups of workers, Issekutz and colleagues in Toronto, and Williams and colleagues in London, both using rabbits. Issekutz and co-workers demonstrated that intradermal injection of various chemotactic factors, including human complement-activated plasma, partially purified C_{5a} des Arg, and the bacterial peptide FMLP not only caused accumulation of ^{51}Cr -labelled PMN's in the skin, but was associated with a rapid increase in vascular permeability, as measured by leak of ^{125}I -labelled albumin from capillaries^{117, 118}. Both PMN accumulation and the permeability increase took place in the first hour following injection, and PMN's continued to accumulate for 3-4 hours. In some animals, neutropenia was induced by intravenous nitrogen mustard. During the period of neutropenia, intradermal injection of activated complement was not associated with either an increase in vascular permeability or red cell leakage¹¹⁸.

A further model which the Toronto group used was intradermal injection of whole *E coli* which, as expected, also caused PMN accumulation, as well as the permeability and

red cell effects produced by chemotactic factors alone¹¹⁹. Their action was independent of complement, but again, neutropenic animals did not show the micro-vascular changes of neutrophil replete animals. Their work therefore suggests that activated PMN's in some way are able to impair the ability of small vessel endothelial lining cells to prevent fluid and protein loss from the circulation.

Williams and co-workers confirmed that complement activation results in the generation of a permeability factor with the physico-chemical characteristics of C_{5a} ¹²⁰ and also found that this permeability factor was inactive in the skin of neutropenic animals¹²¹. Their studies on the mechanism of action of C_{5a} also demonstrated that:-

1. its permeability effects in rabbit skin could not be inhibited by concentrations of anti-histamines which totally abolished histamine-induced skin oedema, suggesting that C_{5a} does not act via skin mast cell activation^{120, 121}.

2. the des Arg form of human C_{5a} , demonstrated to be inactive by the standard guinea pig ileum assay for anaphylotoxic activity, could still increase skin vascular permeability, further proof of a mast cell-independent mechanism¹²².

Their observations and conclusions, however, differ in one important regard from those of Issekutz et al. While Issekutz et al found that both complement-activated plasma and partially purified C_{5a} des Arg each produced significant permeability changes when injected alone, Williams et al could not demonstrate a permeability effect of either C_{5a} or C_{5a} des Arg unless a vasodilator such as prostaglandin E_2

(PGE₂) was injected also¹²¹. This has been termed the 'two mediator hypothesis' of inflammation and proposes that for significant tissue oedema to be produced by an inflammatory stimulus, there must be generation of both an endogenous vasodilator such as PGE₂ and a permeability promoting factor such as C_{5a} des Arg¹²³. Thus a stimulus like whole zymosan (dead yeast cell walls) can cause extravasation of plasma through both complement activation and prostaglandin synthesis. Anti-inflammatory agents which inhibit prostaglandin synthesis (e.g. aspirin, indomethacin etc) suppress the increase in local blood flow and hence block local oedema formation. Exogenous C_{5a} or C_{5a} des Arg, in contrast, produces little extravasation alone, but its action can be greatly potentiated by the addition of PGE₂.

Why there should be such disparity between the findings of the two groups is not immediately obvious. Williams et al appear to have used much purer preparations of C_{5a} and C_{5a} des Arg so it may be that the partially purified material used by Issekutz et al contained an additional vasodilator or permeability promoting factor. Alternatively, the results may simply reflect differences in concentrations of mediators injected. Williams' group used human C_{5a} des Arg at a concentration of $10^{-10} - 10^{-12}$ mol/site in an injection volume of 0.1ml, equivalent to $10^{-6} - 10^{-10}$ M¹²², which, at least at the upper end of that range, is within the effective concentrations of C_{5a} des Arg for PMN activation⁶⁶. Because Issekutz et al used less than pure material, exact concentrations of C_{5a} des Arg cannot be calculated, and their technique for measuring skin response is also different from

that of Williams et al, so quantitative comparisons cannot readily be made. These disparities, although important when considering detailed mechanisms of inflammation, do not alter the two main findings agreed by both groups, which are (1) the C_{5a} derivative, C_{5a} des Arg, increases vascular permeability in rabbit skin when injected locally (2) this effect is absent in neutropenic animals. Some possible explanations for this will now be considered briefly.

D. Possible mechanisms for the effects of PMN's on vascular permeability

For PMN's to increase vascular permeability in the skin, they would require to have one or more of the following effects:-

1. release of a mediator or mediators which would act directly on vascular endothelial cells to produce gaps between them or to impair their integrity. Examples of possible mediators with direct effects on the vessels are the cyclooxygenase product prostaglandin E_2 ¹²⁴, the lipoxygenase product leukotriene B_4 ¹²⁵ and the phospholipid derivative platelet activating factor¹²⁶. Endothelial integrity might also be impaired by toxic oxygen species generated by the PMN oxidase¹²⁷.

2. release of mediators which would act on another cell type to release a second mediator with effects on the endothelium. An example of this is the mast cell stimulation produced by the myeloperoxidase- H_2O_2 -halide system¹²⁸.

3. production of gaps between endothelial cells by direct PMN/endothelial interaction and PMN migration, as suggested by Williams¹²¹.

The work in the **in vivo** section of this study (Chapters 4 and 5) will therefore attempt to answer the following questions:

1. Does activated human complement increase blood flow and vascular permeability in human skin, and if so, which complement components are responsible?
2. Does this effect require an exogenous vasodilator?
3. Does this effect require circulating PMN's?
4. If so, how are they acting?

CHAPTER 2

IDENTIFICATION OF PMN DEGRANULATING ACTIVITY IN ACTIVATED COMPLEMENT AND USE OF A PMN DEGRANULATING SYSTEM TO DETECT COMPLEMENT ACTIVATION.

The work in this chapter was performed in collaboration with Dr J M Davies.

INTRODUCTION

In this chapter, the effects of activated complement on PMN cobalamin binding protein (NCBP) release are studied, and attempts made to identify, both functionally and immunologically, the complement component(s) responsible. The uses of a PMN degranulating system are then explored, with particular reference to the detection of complement activation.

The role of activated complement as an important source of PMN degranulating activity in the circulation was suggested by observations on patients following elective surgery, as discussed in Chapter 1, Section III. Surgery causes a rapid fall in intracellular levels of NCBP and lysozyme, within circulating PMN's taken postoperatively, suggesting that surgery generates a degranulating stimulus intravascularly¹. This stimulus does not appear to arise from the coagulation, kinin, or fibrinolytic pathways³. PMN's incubated *in vitro*,

however, with serum in which the alternative complement pathway has been activated with the yeast cell wall product zymosan, show rapid release of NCBP³. Evidence that activated complement is the PMN degranulating stimulus generated postoperatively is suggested by studies showing that plasma complement is activated by surgery⁹⁶, and also from *in vitro* observations on PMN's taken from postoperative patients. These PMN's show stimulus-specific desensitisation to complement, which works as follows. If normal PMN's are exposed to a degranulating stimulus, washed, and re-exposed to the same stimulus, little further degranulation is seen. If, however, the second stimulus is different to the first, degranulation occurs normally. This phenomenon, known as stimulus-specific desensitisation, is probably due to a temporary loss of specific surface receptors for the initial stimulus due to internalisation of receptor-ligand complexes²⁹. Receptors for other stimuli are largely unaffected. Post-operative PMN's, when challenged *in vitro* with different stimuli, degranulate normally in response to the bacterial peptide FMLP, but are refractory to complement-activated serum¹²⁹. This suggests that they have been exposed to activated complement *in vivo* and that this stimulus can arise within hours of the start of the operation¹.

The following steps in the identification of the complement-derived degranulating activity will now be described and discussed.

1. Optimal method for isolating PMN's and setting up a degranulation system to detect NCBP release.

2. Optimal method for preparing activated complement.
3. Identification of degranulating activity by physical and immunological means.
4. Uses of a PMN degranulation system.

EXPERIMENTAL WORK

For details of suppliers of materials, see Appendix 1.

For details of buffers, see Appendix 2.

GENERAL METHODS

1. Preparation of Neutrophil Suspensions

Venous blood was mixed in plastic universal containers with the anti-coagulant EDTA (ethylene diamine tetraacetic acid-disodium salt). 0.34 mls of 1.34×10^{-1} M EDTA was mixed with 15 mls blood giving a final EDTA concentration of 3.04×10^{-3} M. Two different separation procedures could then be followed.

Method (i)

7.5 mls blood were carefully layered over 3 mls Monopoly Resolving Medium, a Ficoll-Hypaque density gradient with a specific gravity of 1.114, in a 10 ml glass tube. When spun at 400g for 45 minutes at room temperature, separation of plasma, mononuclear cells and PMN's in a single step was seen¹³⁰ (Fig 4).

The PMN layer was harvested and washed twice in cold phosphate buffered saline, (PBS) by resuspending and spinning at 100 g for 10 mins, and finally suspended in 4 mls PBS for counting.

Notes 1. As contamination by red cells was usually very slight, no specific measures were taken to lyse them. 2. To prevent nonspecific release of neutrophil granules during the preparation process, all washing and counting steps were performed in PBS devoid of calcium and magnesium, which are



Fig 4. Separation of blood into red cells, mononuclear cells, neutrophils and plasma in a single step on a Ficoll-Hypaque gradient.

required for degranulation. Only when the degranulation experiment was ready to begin was the required final volume of phosphate buffered saline with albumin (PBS-A), containing calcium and magnesium, added to the cells. Preliminary studies determined the best concentration of albumin to use in this solution (see below).

Method (ii)

Although method (i) of neutrophil preparation worked satisfactorily for most donors, the blood of certain individuals, usually females, did not fractionate satisfactorily by this technique, because the red cells failed to sediment through the Ficoll-Hypaque gradient. Examination of these individuals' blood films and distribution of red cell volumes usually indicated a mild degree of iron deficiency. For such donors, neutrophils could be prepared by the following technique:

EDTA-anticoagulated venous blood was layered on to Lymphocyte Separation Medium, a Ficoll Hypaque mixture with a specific gravity of 1.077, in the ratio of 3 volumes of blood to 1 of LSM and centrifuged at 400 g for 20 minutes at room temperature. This causes separation of the mononuclear cell layer while the red cells and PMN's sediment to the bottom. The mononuclear cell layer was then discarded and the red cell/PMN mixture remixed with the plasma. This suspension was then mixed with 6% dextran in 5% dextrose, (MW = 70,000 Daltons), in a 1:1 ratio in a 30 ml syringe and allowed to stand at an angle of 45° at room temperature for 60 minutes. At the end of this time, most of the red cells had sedimented, and the supernatant containing PMN's and some red cells was

collected. The remaining red cells were lysed by exposure to 0.87% NH_4Cl containing a small amount of NaHCO_3 at 4°C for 5 minutes. The remaining PMN's were washed twice in PBS as before then counted.

2. Preparation of activated complement in whole serum

Three main complement preparations were used as degranulating stimuli (i) whole serum in which the alternative complement pathway had been activated with the yeast cell wall product zymosan, giving zymosan-activated serum (ZAS), (ii) a similar preparation in which conversion of C_{3a} and C_{5a} to their des Arg forms had been prevented by the addition of epsilon-amino caproic acid (EACA), giving EACA-ZAS, (iii) a partially purified preparation of EACA-ZAS containing the fraction which had passed through a 30,000 dalton molecular exclusion filter (low molecular weight fraction - LMW fr, see Exp 7 below).

Other complement-activated sera used in the identification of the degranulating activity were heated, C_5 depleted or chromatographed (see below).

(i) Zymosan-activated serum (ZAS)

Venous blood from three or four donors was allowed to clot in glass tubes for 60 minutes at room temperature, and the serum separated by centrifugation at 1500g for 10 minutes. The serum was pooled and mixed with boiled, washed zymosan (final concentration 2mg/ml of serum) on a rotator at 37°C for 60 minutes. To remove the zymosan, the serum was centrifuged at 1500g for 15 minutes and passed through a 0.8μ filter. 1.0 ml aliquots of the ZAS were stored under liquid nitrogen.

(ii) Zymosan-activated serum containing EACA (EACA-ZAS)

To pooled serum, EACA was added and thoroughly dissolved, and the serum complement activated with zymosan as before. Preliminary studies compared various EACA concentrations (see below), following which 0.5M was used in all experiments.

3. The degranulation system

The general method of this type of experiment was the same no matter what the stimulus, except that in some experiments the cells were exposed to the fungal metabolite cytochalasin B, which although unable to degranulate PMN's on its own, both accelerates and amplifies degranulation in response to most stimuli. The general outline is illustrated in Fig 5. Where a drug inhibitor of degranulation was being tested, it was usually added prior to the cytochalasin B.

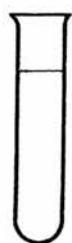
Details of method

The PMN's were kept at room temperature, in an undiluted form in PBS till the start of the experiment, when they were diluted to the required volume with PBS-A. Each experimental condition was performed in duplicate, and the final reaction volume in each tube was 1.0ml. To start the experiment, 2×10^6 PMN's in PBS-A were pipetted into LP3 polypropylene tubes and prewarmed at 37°C for 5 minutes. If cytochalasin B was used, 0.1 ml of a $50 \mu\text{g/ml}$ solution was then added giving a final concentration of $5 \mu\text{g/ml}$, and the cells incubated with it for 5 minutes at 37°C . 0.1 ml of the stimulus was then added and the incubation continued for a further 5 minutes, after which degranulation was terminated by centrifugation at 4°C at 1500 g for 10 minutes. The supernatants were removed

Fig 5.

DEGRANULATION SYSTEM

2×10^6
neutrophils in
PBS 0.1% albumin



Cytochalasin B $5\mu\text{g/ml}$ in DMSO

5 minutes at 37°C



Stimulus - ZAS
low MW fraction

5 minutes at 37°C

Centrifuge at 4°C



Supernatant assayed for
cobalamin binding protein

Results expressed as a percentage of the total intracellular
cobalamin binding protein obtainable by sonication of the cells.

and stored at -20°C till the NCBP assay was performed. Where cytochalasin B was not included, the incubation time with the stimulus was lengthened appropriately (see Chapter 3).

Calculation of results

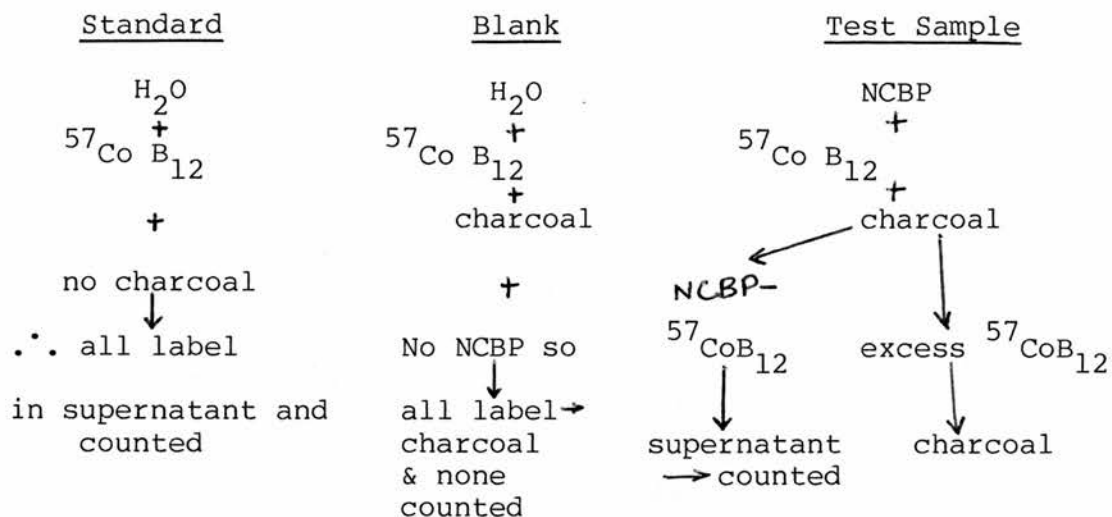
Each experiment contained a blank (PMN's in PBS-A with cytochalasin B if appropriate), and a sonicate, obtained by sonicating a measured aliquot of PMN suspension containing 2×10^6 cells, at setting 5 on a cell sonicator for 2 minutes. These conditions have been previously shown to give maximal release of granule contents³. The amount of NCBP in the sonicate was therefore presumed to be 100% of the intracellular level. Results were therefore calculated as follows:-

$$\% \text{ NCBP release} = \frac{\text{mean of duplicates} - \text{blank}}{\text{sonicate}} \times \frac{100\%}{1}$$

4. Assay of neutrophil cobalamin binding protein

(unbound vitamin B_{12} binding capacity)¹³¹

This assay is based on the binding of vitamin B_{12} labelled with the γ emitter $^{57}\text{cobalt}$ ($^{57}\text{CoB}_{12}$), to the NCBP. Excess $^{57}\text{Co B}_{12}$ is added, and any which has not bound to NCBP is removed by adsorption on to protein-coated charcoal¹³². The $^{57}\text{Co B}_{12}$ which has bound to NCBP is not taken out by the charcoal, and appears in the supernatant which is collected for counting. Each batch of samples contains a standard (no charcoal or NCBP) and a blank (no NCBP). Thus:



Thus the number of counts in the supernatant is directly proportional to the concentration of NCBP present.

Notes

1. Because the assay depends on the availability of vitamin B_{12} binding sites on the binding protein, only CBP which has not already bound some B_{12} can be counted. PMN cobalamin binding protein has no B_{12} bound to it, and can thus be measured in this way. Hence results are expressed as vitamin B_{12} binding capacity i.e. the amount of vitamin B_{12} (ng/ml) which could be bound by the CBP present.
2. It follows that all test samples and reagents used must be free from extraneous vitamin B_{12} or CBP, which sometimes occurs as a contaminant in preparations of albumin and other proteins. Where this is unavoidable, e.g. when whole complement-activated serum is used as the degranulating stimulus, the serum CBP is measured and subtracted, since at a final serum concentration of 10%, the serum CBP may be as much as 25% of the total³.
3. If all the supernatant is removed from cells which have

secreted, and the cell pellet then sonicated, the total NCBP in supernatant plus cell pellet is the same as in a sonicate of unstimulated cells³. Thus the cells do not appear to rapidly resynthesise NCBP as it is secreted.

4. The charcoal used to remove the unbound $^{57}\text{Co B}_{12}$ must be coated with a protein to reduce the pore size and thereby exclude any $^{57}\text{Co B}_{12}$ -NCBP complexes. Initial experiments used bovine serum albumin although later, bovine haemoglobin was used. This had certain advantages:-

- (i) there was less CBP contamination
- (ii) its binding to the charcoal was more stable, allowing more samples to be processed in one batch.

The overall results were not affected by whether albumin or haemoglobin was the protein used.

Details of NCBP assay (After Lau et al 1965)¹³³.

To save reagents, smaller volumes were used, so to achieve greater accuracy, positive displacement pipettes were used throughout.

1. 50 μl of each sample was pipetted into an LP₃ polypropylene tube, 50 μl distilled water being used for the standard and blank tubes.
2. Using an Eppendorf multipipette, 100 μl $^{57}\text{CoB}_{12}$ was added to all the tubes, which were vortexed and incubated at room temperature in the dark (because of photolability of the $^{57}\text{Co B}_{12}$) for 30 minutes.
3. Using the multipipette 200 μl protein-coated charcoal was added to all tubes except the standard, which received 200 μl distilled water.
4. All tubes were vortexed and immediately centrifuged at 1500

g at 4°C for 7 minutes.

5. 200 μ l of supernatant was removed from each tube and placed in a separate tube for counting. The tubes were counted for 4 minutes each in a gamma counter.

| Volumes (μ l) | distilled water | plasma | $^{57}\text{Co-B}_{12}$ | Charcoal |
|--------------------|-----------------|--------|-------------------------|----------|
| standard (A) | 250 | 0 | 100 | 0 |
| blank (B) | 50 | 0 | 100 | 200 |
| test (C) | 0 | 50 | 100 | 200 |

\therefore total volume = 0.35 ml.

Calculation of results:-

$$\text{Unbound B}_{12} \\ \text{binding capacity (ng/l)} = \frac{C-B}{A} \times \text{conc. } ^{57}\text{Co-B}_{12} \times \text{plasma dilution}$$

provided that $^{57}\text{Co-B}_{12}$ of specific activity 15 $\mu\text{Ci}/\mu\text{g}$ of B_{12} is used, at a concentration of 2000 ng/l. (see below).

Preparation of reagents

1. $^{57}\text{Co-B}_{12}$ (^{57}Co -cyanocobalamin)

A 1 ml vial of high specific activity $^{57}\text{Co-B}_{12}$ was diluted to a concentration of 2000 ng/l in 0.9% benzanol in distilled water, and stored at 4°C.

2. Protein coated charcoal (made fresh daily).

0.5g activated charcoal was added to 20 ml of either bovine haemoglobin (2.5g/l in distilled water) or bovine serum albumin (6g/l in distilled water). After mixing on a magnetic stirrer for at least five minutes, the charcoal was ready for use, and could be stored at 4°C for the rest of the working day.

Experiment 1 Comparison of PMN's prepared by the single step and two step preparation methods.

As preparation of PMN's by the single step method was considered a departure from standard techniques, PMN's prepared by the 2 methods were compared for yield, purity, viability, NCBP levels, and response to stimuli.

A. PMN counting and calculation of yield.

Method PMN suspensions were counted by diluting 20 μ l of suspension in 10 mls isotonic saline (Isoton II). Red cells were lysed by the addition of 4 drops of Zaponin. Counts were performed on a Coulter DN counter.

Yield was calculated from the final PMN count and the donor's peripheral blood PMN count obtained by performing a differential count of 100 leucocytes on a standard blood film.

$$\text{Then yield} = \frac{\text{actual count}}{\text{expected count}} \times \frac{100\%}{1}$$

For example, from 1 donor, 44×10^6 PMN's were obtained from 30 mls blood, in which the initial count was $4.24 \times 10^9/l$.

$$\begin{aligned} \therefore \text{yield} &= \frac{44 \times 10^6}{\frac{4.24 \times 10^9}{33.33}} \times \frac{100}{1} \% \\ &= \frac{44 \times 10^6}{1.27 \times 10^8} \times \frac{100}{1} \% \\ &= \frac{44 \times 10^8}{1.27 \times 10^8} \% \\ &= \underline{34.65\%} \end{aligned}$$

Results For most experiments, yields of between 30 and 40% were obtained. There was no difference in yield between the two methods.

B. PMN Purity

Method A slide of the PMN suspension was made by passing 0.1 ml of a 0.2×10^6 /ml dilution of cells through a cytospin. The slide was dried and stained with Diff-Quik, a variation of the Leishman stain. Two hundred cells were counted, and a differential count calculated.

Results PMN suspensions were usually at least 95% pure, the main contaminant being eosinophils, with the occasional lymphocyte and monocyte, and there were no significant differences between PMN's prepared by the two methods.

C. PMN Viability

Method Viability was assessed using the fact that viable PMN's can exclude the dye Trypan Blue, while dead cells take it up passively. Three drops of a 0.3×10^6 /ml cell suspension were mixed with 1 drop of a 5% solution of Trypan Blue in isotonic saline on a microscope slide. After 5 minutes, the proportion of stained cells was counted.

Results

Greater than 99% of PMN's were viable as assessed by this method, no matter by which method they were prepared.

D. NCBP levels and release

Previous studies had compared behaviour of PMN's prepared by the new single step method using monopoly resolving medium to those separated by the traditional 2 step method of mononuclear cell removal followed by dextran sedimentation of red cells. In these studies, no differences in PMN random mobility or chemotaxis were found¹³⁴, but no comparison of degranulation had previously been made.

Method PMN's prepared by each of the two methods were

challenged with ZAS as described above in the general methods.

Results Figure 6 shows that both stimulated and unstimulated cells separated by the two methods behaved identically. Levels of NCBP in sonicated cells were within 10% of each other, suggesting that the new method does not cause excessive granule loss during preparation.

Experiment 2 Effect of the albumin concentration in PBS-A on NCBP release in response to ZAS.

Method As exposure of PMN's to albumin alone can cause aggregation and hence possibly granule release, control tubes had to include albumin to correct for that present in serum-based stimuli. A comparison of NCBP release in response to ZAS was therefore made in PBS containing albumin concentrations of zero, 0.1% and 1.0%, using the general method.

Results Measurement of NCBP in sonicated PMN's was unaffected by albumin concentration, but NCBP release from both unstimulated and stimulated cells was influenced by it (Fig 7). Release from unstimulated cells increased as the albumin concentration rose, so that at an albumin concentration of 1.0%, release was nearly 30% of the sonicate. There was no difference in NCBP release in response to ZAS between cells containing 0.1% albumin and those containing 1% albumin. Therefore all subsequent experiments were performed in PBS containing 0.1% albumin.

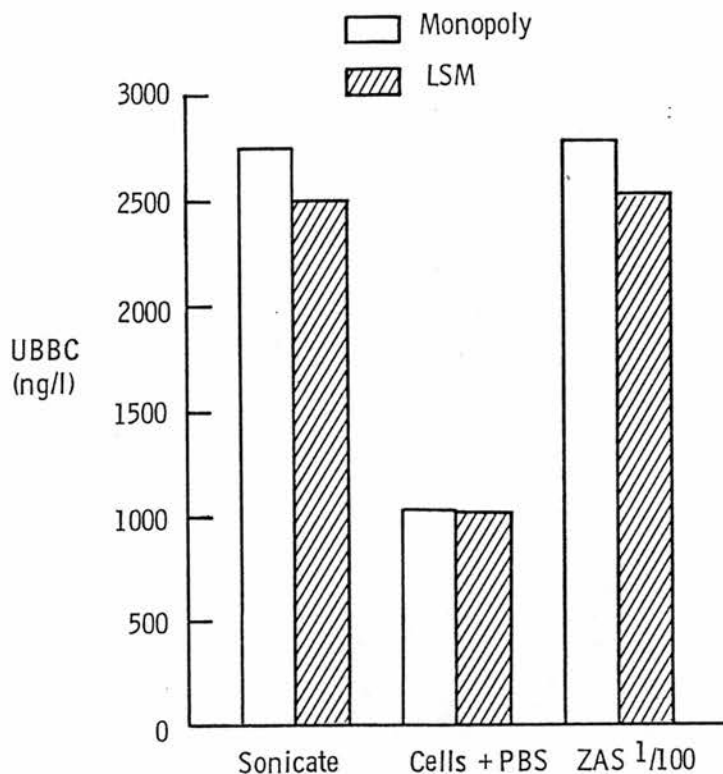


Fig 6. Release of NCBP, measured as unbound vitamin B₁₂ binding capacity (UBBC), from sonicated PMN's, PMN's in PBS only, and PMN's stimulated with ZAS (1%). Comparison is made of responses of cells prepared either on monopoly or on lymphocyte separation medium.

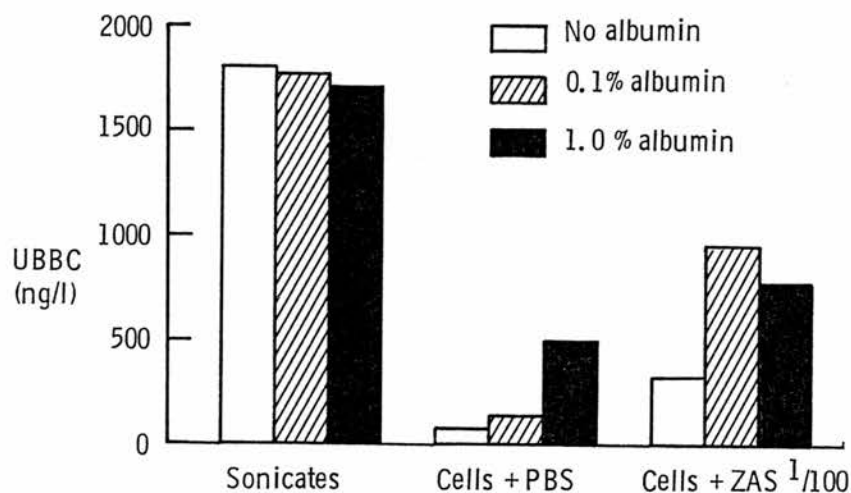


Fig 7. Effect of different albumin concentrations on release of NCBP (measured as UBBC) by sonication, in resting cells, in PBS and in cells stimulated with ZAS 1/100.

Experiment 3 Effect of the concentration of epsilon amino caproic acid (EACA) on the generation of degranulating activity in EACA-ZAS.

Method Although inhibition of conversion of C_{5a} to C_{5a} des Arg by the plasma enzyme carboxypeptidase B requires high concentrations of EACA, it has been suggested that excessively high concentrations can actually block C_{5a} formation¹³⁵. A preliminary comparison was therefore made of the degranulating activity in dilutions of EACA-ZAS prepared in the presence of different EACA concentrations, namely 0.1M, 0.5M and 1.0M, using the general method.

Results There was no significant difference between degranulating activity generated in the presence of 0.5M and 1.0M EACA (Fig 8). The reduced amount of degranulating activity generated in the presence of 0.1M EACA suggests that this concentration of EACA is insufficient to prevent conversion of C_{5a} to its less potent des Arg form. Therefore an EACA concentration of 0.5M was used in all subsequent preparations of EACA-ZAS.

Note on release from unstimulated cells.

Cytochalasin B treated cells, when suspended in PBS-A, will secrete a small amount (<5%) of their NCBP during the incubation period, probably due to activation during the separation process³. In early experiments, this release was noted to be excessively high, up to 25-30% of sonicate values. This was eventually discovered to be due to the stimulation of the cells by micro-crystals of calcium and magnesium phosphate, formed when calcium and magnesium chloride were added to the cell suspensions in PBS just prior to the start of the

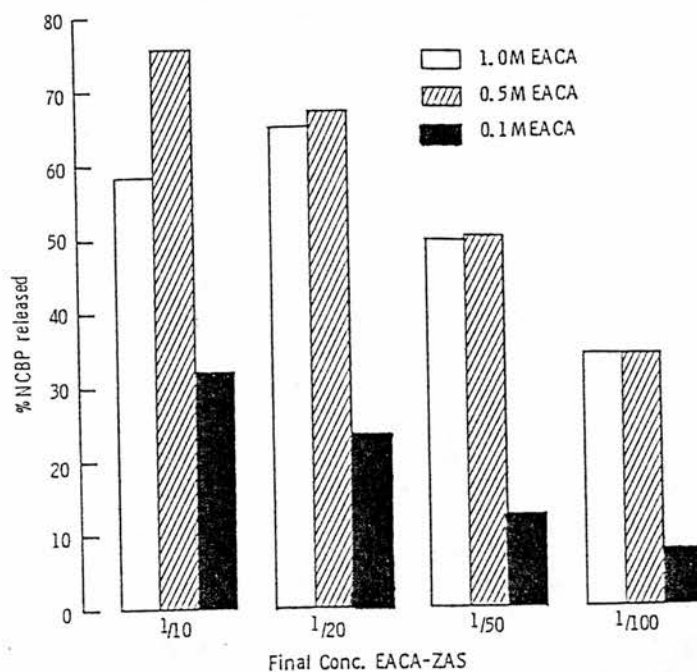


Fig 8. Effect of different concentrations of EACA during complement activation on the generation of NCBP-releasing activity in EACA-ZAS.

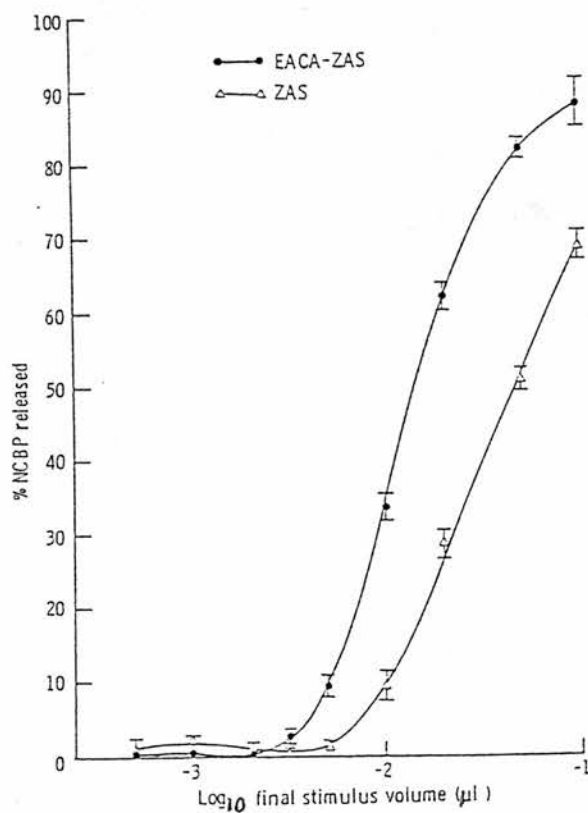


Fig 9. NCBP release from cytochalasin B treated PMN's by dilutions of ZAS and EACA-ZAS. (mean \pm SEM, n = 10).

experiment. The method was altered so that PBS-A was prepared as described in Appendix 2, so that the calcium and magnesium salts were thoroughly dissolved before addition to the cells. When this method was adopted, secretion of NCBP by unstimulated cells fell to less than 5% of sonicate values.

Experiment 4 Release of NCBP from PMN's by zymosan-activated serum with and without EACA.

Method Dilutions of ZAS and EACA-ZAS (containing 0.5M EACA) were added to cytochalasin B treated PMN's as above.

Results Both ZAS and EACA-ZAS produced dose dependent NCBP release (Fig 9). Serum activated in the presence of EACA was approximately 5 times more potent a stimulus than non-EACA-treated serum. The dose-response curve obtained for EACA-ZAS begins to flatten off at the top end, while that for ZAS does not. It is likely that if the serum could be concentrated further, both curves would flatten off. However, the highest concentration (10^{-1}) was obtained by adding neat serum to the cell suspension. No release above that obtained with PBS-A alone was seen with 0.5M EACA alone or with unactivated serum.

Experiment 5 The effects of heat-treated sera, and EDTA and EGTA plasmas on NCBP release.

Method In order to inactivate complement components, pooled serum was heated at 56°C for 30 minutes prior to, or following, zymosan activation. To assess the relative importance of the classical and alternative complement pathways, plasma was obtained from venous blood containing either ethylene-diamine

tetraacetic acid (EDTA), which chelates calcium and magnesium ions, or ethylene-bis-(oxy-ethylene-nitrile) tetraacetic acid (EGTA), which chelates calcium ions only (final concentration of each = 10mM). EDTA, by chelating both calcium and magnesium ions, inhibits both classical and alternative complement pathways, while EGTA, chelating only calcium ions, inhibits only the classical pathway. To allow degranulation to occur in response to these sera, excess calcium and magnesium had to be added so that the chelating effects could be overcome. Therefore, calcium and magnesium chloride were added to a final concentration of 12 mM, estimating that between 1 and 2 mM of these would remain in the ionised form.

Results Heating serum to 56°C for 30 minutes prior to zymosan activation prevented the generation of degranulating activity, but sera heated after zymosan activation showed little loss of activity (Fig 10).

The inhibitory effect of EDTA, but not EGTA, suggested that an intact alternative complement pathway, requiring Mg^{++} but not Ca^{++} , is necessary for generation of degranulating activity (Fig 10).

Experiment 6 Column chromatography of EACA-ZAS.

Method EACA-ZAS was passed downwards on a Sephadex G200 column, 28 x 300 mm, using 0.4M PBS containing 0.01M EDTA at room temperature. Fractions were collected, and 0.5 ml aliquots of each fraction were tested for their ability to stimulate NCBP release.

Results A single broad peak of degranulating activity was obtained, representing material with a molecular weight of less

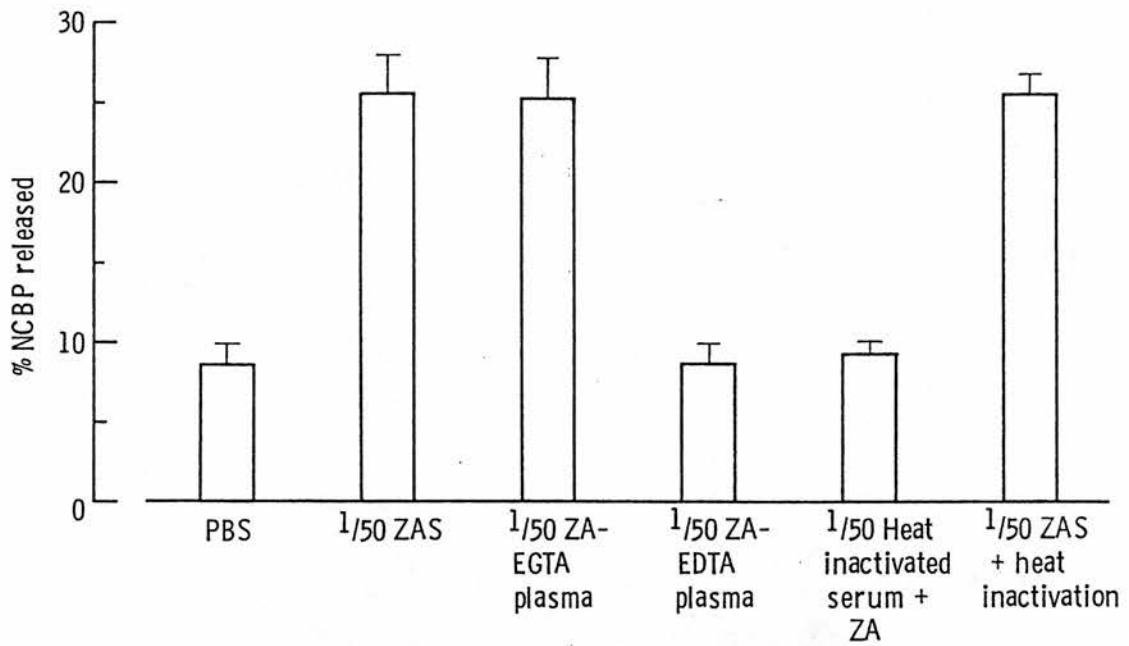


Fig 10. NCBP release from cytochalasin B treated PMN's by various complement activated sera and plasmas.

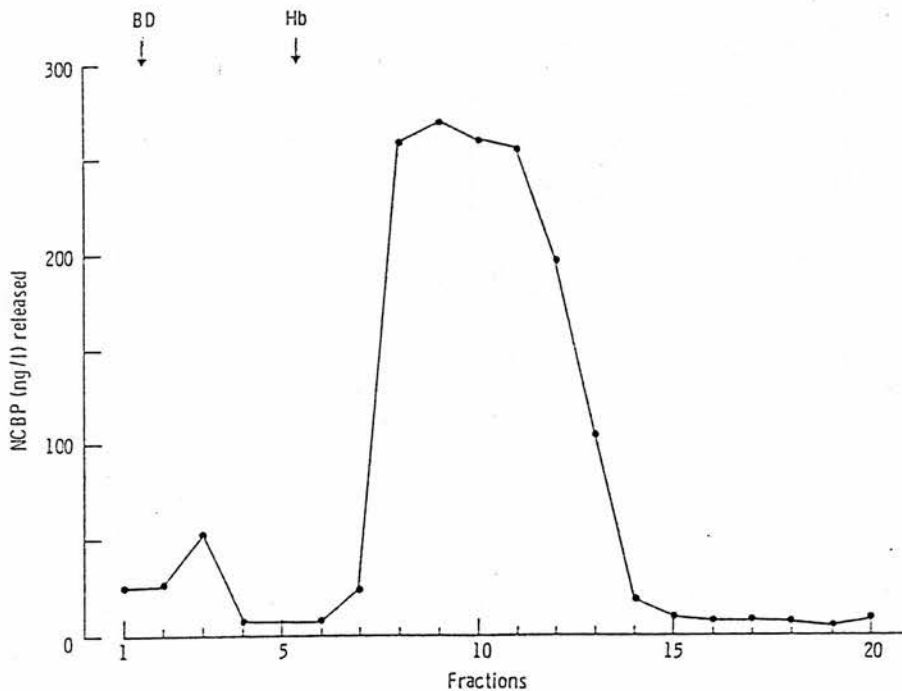


Fig 11. NCBP release from cytochalasin B treated PMN's by fractions of EACA-ZAS from a Sephadex G 200 column.

than that of the marker protein haemoglobin (MW 64,500 Daltons, Fig 11).

Experiment 7 Use of a micropartition membrane to further identify and partially purify the degranulation activity in EACA-ZAS.

Method The sample to be fractionated was layered in 1ml aliquots over a 30,000 Dalton exclusion ultrafiltration membrane (Fig 12) and centrifuged for 30 minutes at 2000g at room temperature. This membrane excludes >99.9% albumin, and it can therefore be assumed that the ultrafiltrate is of low molecular weight, although not all small molecules in the original solution are able to pass through, due to 'clogging up' of the membrane pores by large molecules.

Results When the chromatography peak of degranulating activity in EACA-ZAS was applied to the micropartition membrane degranulating activity was found only in the low molecular weight fraction (ultrafiltrate) (Fig 13).

When whole EACA-ZAS was applied to the 30,000 Dalton exclusion filter, degranulating activity again appeared in the ultrafiltrate. When aliquots of a batch of ultrafiltrate were applied to cytochalasin B treated PMN's, dose-dependent NCBP release was obtained (Fig 14). As with whole ZAS and EACA-ZAS, the dose-response curve does not flatten off at higher concentrations, although this may have been demonstrated had it been possible to concentrate the stimulus further.

When ZAS prepared in the absence of EACA was ultrafiltered, very little degranulating activity appeared in the ultrafiltrate, in contrast to that obtained with EACA-ZAS.



Fig 12. The micropartition membrane system (membrane cut off point 30,000 Daltons) used to prepare the low molecular weight fraction of EACA-ZAS.

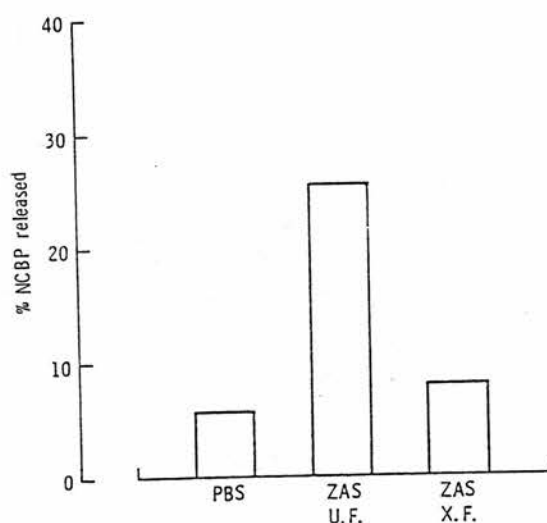


Fig 13. NCBP release from cytochalasin B treated PMN's by the peak of degranulating activity from sephadex chromatography (Fig 11) separated into high (X.F) and low (U.F) molecular weight fraction.

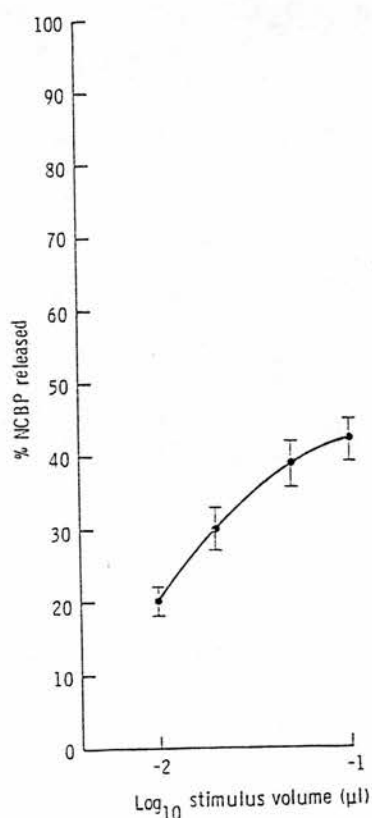


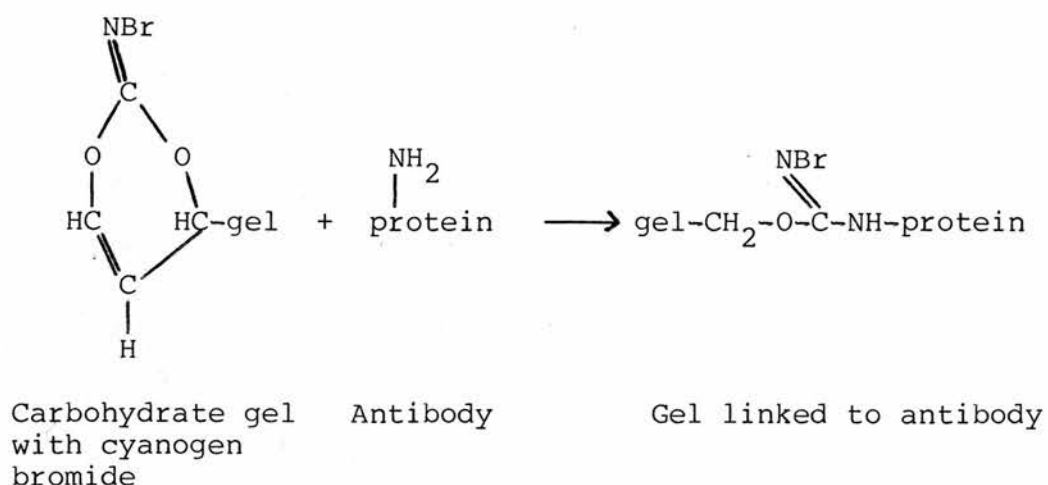
Fig 14. Release of NCBP from cytochalasin B treated PMN's by the low molecular weight fraction of EACA-ZAS. (Mean \pm SEM, n = 10).

Possible reasons for this are discussed below.

Experiment 8 Preparation and assessment of an affinity gel

to remove C_5 and products of C_5 from sera.

To assess the role of C_5 derived products in NCBP release serum was depleted of C_5 prior to or following zymosan activation using an anti- C_5 antibody bound to a carbohydrate gel. When serum, or any other mixture of proteins, is exposed to such a gel, the appropriate antigen, in this case C_5 , will bind to the antibody while other antigens remain unbound, allowing specific removal of a single protein in a mixture. In some instances, the bound protein can be eluted from the gel by a change in pH or ionic strength, although in the case of C_5 this is not possible without damage to the molecule¹³⁶. In any case, the bound protein must be washed from the gel before further use. For linking any ligand with NH_2 groups, including antibodies, to a gel, cyanogen-bromide-activated sepharose 4B is the gel used¹³⁷. The coupling is probably via imidocarbonate groups:-



Methods (from handbook of Pharmacia Fine Chemicals Ltd, Upsalla).

The antibody bound was a polyclonal sheep anti-human C₅ antiserum (see Appendix 1 for details of all antibodies).

(i) Preparation of the gel

Since 1 gram of gel binds 10 mg of protein, the exact protein concentration in the antiserum was calculated from its optical density at 280 nanometers (OD₂₈₀), knowing that an IgG solution of 1 mg/ml has an OD₂₈₀ of 1.4.

OD₂₈₀ of a 1 in 30 dilution of sheep anti-human C₅ = 0.526.

$$\therefore \text{protein concentration} = \frac{30 \times 0.526}{1.4} \text{ mg/ml}$$

$$= 11.25 \text{ mg/ml}$$

Therefore 5 ml antiserum and 5 g freeze dried gel were used. The gel was first washed with 1 litre 1 mM HCl (pH 2.5) on a sintered glass funnel (64) to allow reswelling and to remove the dextran and lactose which are added to the gel as stabilisers. The gel was then washed with coupling buffer (pH 8.3) (see Appendix 2 for details of buffers). The antiserum

was dissolved in coupling buffer to a final protein concentration of approximately 2 mg/ml. This was then mixed with the gel in a cell culture flask on a rotator overnight at 4°C to allow binding, then washed with coupling buffer. To block any remaining active groups on the gel which might nonspecifically bind proteins in samples, the gel was mixed with 1.0 M diethanolamine (pH = 8.0) for two hours at room temperature, then washed again alternately with 1 litre aliquots of 0.1 M acetate buffer containing 1.0 M NaCl (pH = 4.0), and 0.1 M borate buffer containing 1.0 M NaCl (pH = 8.0) to a total of 4 litres of each. The gel was finally washed with 1 litre PBS and two drops sodium azide added as a preservative. The gel was packed into a 10 ml column, covered with a permeable disc, filled to the top with PBS and stored at 4°C.

(ii) Use of the anti-C₅ gel to remove C₅ and C₅ conversion products from sera

A. Removal of C₅ by incubation of serum with the anti-C₅ gel.

A portion of gel-antibody was added to 3 mls of neat unactivated serum in a 10 ml glass tube, and mixed on a rotator at room temperature for 30 minutes. The serum was collected by passage through a serum harvester (Fig 15). In an attempt to wash the bound C₅ off the gel, the gel was washed twice for 20 minutes with glycine HCl to produce 'eluates' which were collected in the same way as the serum. The gel was then washed 4 times in PBS, in which it was stored. This method was also applied to EACA-ZAS and ZAS, and to the low molecular weight fraction of EACA-ZAS. Serum and eluates were examined for C₃ and C₅ (see Experiment 9).

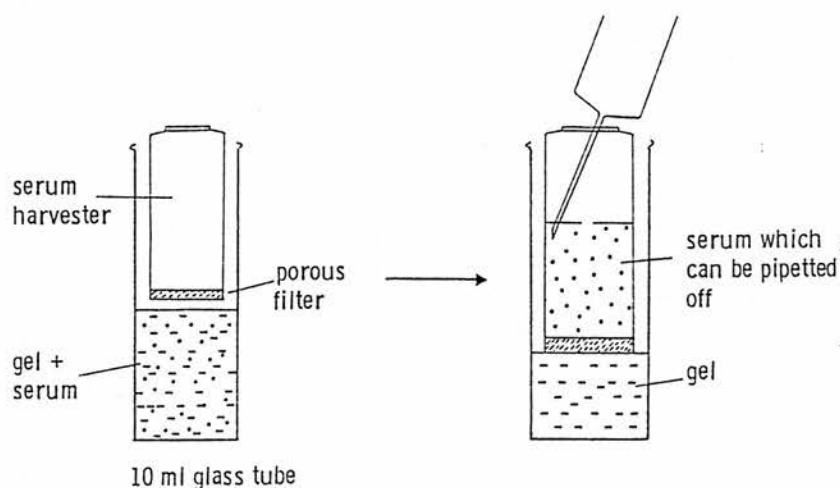
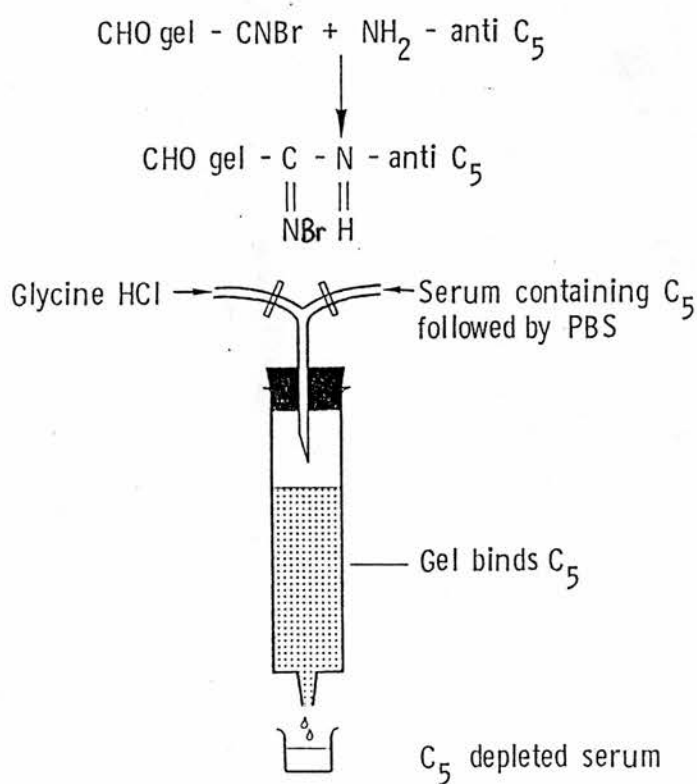


Fig 15. Preparation of C_5 -depleted serum by incubation with anti- C_5 gel.



Gel washed with glycine HCl to allow further binding

Fig 16. Preparation and use of Anti- C_5 affinity column.

B. Removal by passage over a column of gel.

Three mls neat pooled unactivated serum were layered over the filter disc and allowed to flow into the gel by gravity alone (Fig 16). When all the serum had entered the gel, it was washed through with PBS and 3 ml fractions collected at the bottom. The protein concentration in each fraction was calculated from its OD_{280} , and fractions were collected until the OD_{280} was less than 0.050. Most of the protein appeared in 2 or 3 consecutive fractions, usually numbers 3-5, when dilutions had to be made to measure the OD_{280} . The column was then washed with glycine HCl (pH 2.8) and further 3 ml fractions collected.

Results These are the results of a typical preparation.

| fraction No | OD_{280} | protein conc (g/l) |
|-------------|------------|--------------------|
| 1 | 0.103 | <0.175 |
| 2 | 0.890 | 0.8 |
| 3 | 20.10 | 25.00 |
| 4 | 33.98 | 33.00 |
| 5 | 1.621 | 2.85 |
| 6 | 0.308 | 0.35 |
| 7 | 0.125 | 0.175 |
| 8 | 0.102 | <0.175 |
| 9 | 0.064 | <0.175 |
| 10 | 0.055 | <0.175 |

etc.

When the gel was washed with glycine HCl, there appeared to be a small amount of protein in one fraction (No 4), suggesting that the C_5 had been washed off:-

| fraction No following glycine HCl | OD ₂₈₀ | protein conc (g/l) |
|--------------------------------------|-------------------|--------------------|
|--------------------------------------|-------------------|--------------------|

| | | |
|---|-------|--------|
| 1 | 0.132 | <0.175 |
| 2 | 0.164 | 0.175 |
| 3 | 0.113 | <0.175 |
| 4 | 0.63 | 0.60 |
| 5 | 0.117 | <0.175 |
| 6 | 0.046 | <0.175 |

The gel was then washed thoroughly with PBS, and the eluate neutralised with NaOH 1.0 M to pH 7.4.

The above process was followed for unactivated serum, and for both EACA-ZAS and ZAS containing the C₅ conversion products C_{5a} and C_{5a} des Arg respectively. The low molecular weight fraction of EACA-ZAS was also treated identically. All preparations were examined for C₃ and C₅ (see Experiment 9).

Experiment 9 Immunological examination of normal serum, ZAS, and sera and eluates from the anti-C₅ gel.

In order to examine the effects of exposure to the anti-C₅ gel on complement activated sera, the immunological properties of normal serum and ZAS first had to be defined. Both immunoelectrophoresis (IEP) and Ouchterlony immunodiffusion were used to attempt to identify C₃, C₅ and their products in these preparations.

Methods

(A) Ouchterlony Immunodiffusion

Principle

Antigen and antibody are placed in wells cut in agar. From there they diffuse towards each other, and precipitation lines are formed if the appropriate antigen is present. Since lines between antibody and a single antigen always form at the

same distance into the gel, lines of identity will form round the central antibody-containing well if the same antigen is present in several wells (Fig 17). Additional antigens to which the antiserum also reacts will form 'spurs' beyond the lines of identity.

Details of Method

4 mls agar (1%) was poured into a 3 inch round plastic Petri dish, and when cool, holes were cut in the agar. 20 μ l of a 1 in 5 dilution of sheep anti-human C₅ antiserum were placed in the centre well, and 20 μ l of the following sera were placed in the other 6 wells.

1. neat untreated human serum
- 2 & 3. the high protein fractions 3 & 4 (pooled) from column-treated serum (not zymosan activated)
4. the eluted protein fraction (fraction 4 post glycine-HCl)
- 5 & 6. fractions 6 & 7 post glycine HCl (Low OD₂₈₀ controls).

Further plates were prepared using either sheep anti-human C₅ or rabbit anti-human C₃ (Blood Transfusion service, Bristol) (both 1 in 5 dilutions) in the centre well and the following preparations in the outer wells:

1. neat untreated human serum
- 2 & 5. high protein fractions (pooled) after passage of pooled unactivated serum
3. serum incubated with the anti-C₅ gel as above
4. glycine HCl eluate from column
6. glycine HCl wash from incubation method.

The plates were placed in a moist cupboard at room temperature for 18 hours to allow diffusion to occur.

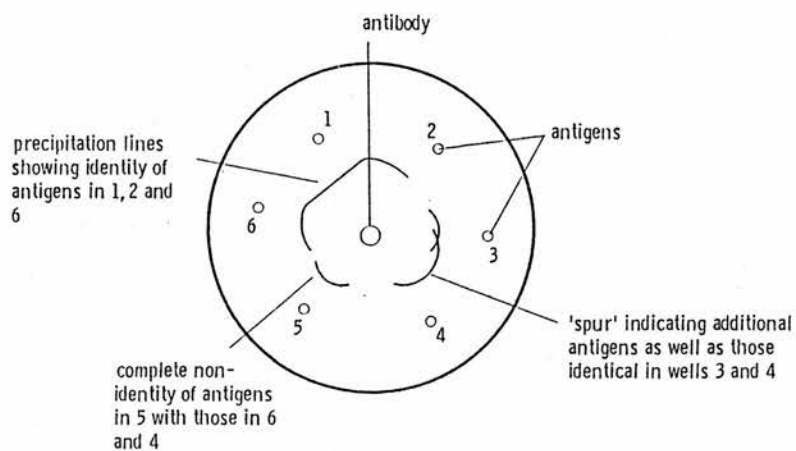


Fig 17. Ouchterlony immunodiffusion.

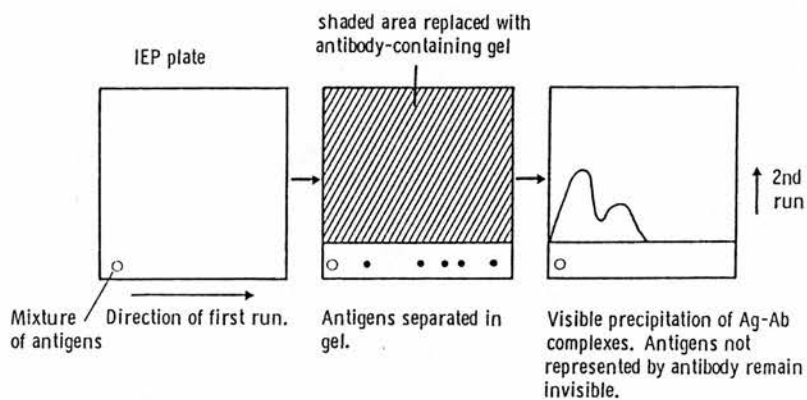


Fig 18. Two-directional immunoelectrophoresis (IEP).

(B) Two-dimensional immunoelectrophoresis (IEP)

Principle

This separates a mixture of protein antigens on the basis of electrophoretic mobility on a gel. The plate is then turned through 90° , and a second electrophoresis performed into a gel containing antibody. Peaks of precipitation form where antibody and antigen meet, and the various antigens precipitating with the antibody can be identified by their position on the plate (Fig 18).

Details of Method

1% agarose in barbitone buffer (pH 8.6) was poured on to a 5 cm square glass plate to a depth of 1.5 mm and allowed to cool. A 1.5 mm diameter well was cut in the bottom left corner and 4 μ l serum added. The serum proteins were separated by electrophoresis at 8 volts/cm in a 4°C cold room for 60 minutes.

The gel not containing proteins was scraped off the plate and 3.0 cm³ of 1% agarose containing either 90 μ l rabbit anti-human C₃ (Blood Transfusion Service, Bristol) or 40 μ l sheep anti-human C₅ layered on. The separated proteins were then electrophoresed into the antibody-containing gel at 5 volts/cm for 3 hours at 4°C . Visible precipitates could then be seen. To make a permanent record unprecipitated protein was removed by covering the plates with wet filter paper and leaving overnight. The plates were then dried with filter paper and stained with 0.5% Coomassie Blue in a solvent mixture of ethanol, distilled water, glacial acetic acid (45:45:10). Excess stain was washed out with solvent, and the plates thoroughly dried.

Results

A. Normal serum, ZAS, and EACA-ZAS.

(i) C_3

When the IEP appearances of either ZAS or EACA-ZAS were compared with those of normal unactivated plasma, an obvious C_3 peak persisted, representing either unconverted C_3 or large C_3 conversion products with similar mobility and antigenic structure to C_3 (Fig 19). There were also additional peaks representing small C_3 conversion products with faster mobility. The polyclonal anti- C_3 antisera obviously contained some activity towards these fragments, likely to be C_{3c} and C_{3d} .

(ii) C_5

On IEP, an obvious C_5 peak was detected in normal serum, with or without EACA, and in ZAS, with or without EACA (Fig 19). Unlike the findings with anti- C_3 , however, neither ZAS nor EACA-ZAS contained extra peaks of small C_5 conversion products, suggesting that they did not form visible precipitates with the anti- C_5 antibody. IEP of the low MW fraction of EACA-ZAS produced no detectable peaks at all.

Immunodiffusion studies using anti- C_5 diluted 1 in 5 revealed a single line of identity linking normal serum with and without EACA, and ZAS with and without EACA. There were no additional lines which might have represented C_5 conversion products.

As with IEP, immunodiffusion studies of the low MW fraction of EACA-ZAS produced no visible lines at all.

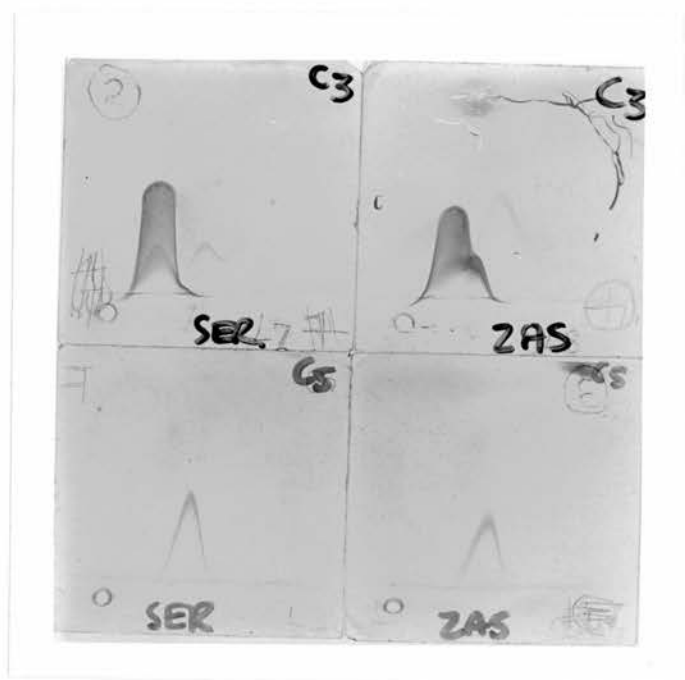


Fig 19. Immunoelectrophoresis of normal serum (2 left squares) and ZAS (2 right squares) into gel containing anti-C₃ (top 2 squares) or anti-C₅ (bottom 2 squares).

When anti-C₃ is used, activation of serum by zymosan produces 2 obvious additional peaks, a small 'hump' to the right of the main C₃ peak, and a faint peak to the right of and further out than the main peak.

When anti-C₅ is used, however, activation of serum by zymosan produces no similar additional peaks.

B. Sera which had been treated with the anti-C₅ gel.

(i) C₃

To ensure that the key complement component C₃ was not also being removed by the anti-C₅ gel, normal serum, serum after treatment with gel, and eluates from the gel were all examined with anti-C₃ by IEP and immunodiffusion.

Similar findings were obtained with both methods. Normal serum and gel-treated serum both gave clear precipitation lines with anti-C₃, suggesting that little, if any, C₃ is removed by the anti-C₅ gel. Eluates from the gel gave no precipitation lines with anti-C₃.

(ii) C₅

Both IEP and immunodiffusion gave similar results. While normal human serum gave strong precipitation lines in both detection methods, no precipitation lines were seen with serum which had been exposed to the anti-C₅ gel, either by incubation or on the column. This suggests that either method completely removes C₅ from normal human serum, or that any residual C₅ is present in concentrations below the limits of detection of these methods.

When eluates from the gel were examined, no precipitation lines were seen either by IEP or immunodiffusion. The most likely explanation for this is that the elution process using glycine HCl destroys the antigenic structure of the molecule, as previously found¹³⁶.

As C₅ conversion products in neither ZAS, EACA-ZAS nor low MW fraction could be detected by IEP or by immunodiffusion, their possible removal by gel-bound anti-C₅ antibody could not be detected by these techniques. Sera so treated were

therefore merely tested for degranulating activity.

Experiment 10 Release of NCBP from PMN's by ZAS prepared from sera depleted of, or deficient in, C_5 .

This experiment was designed to assess the importance of C_5 products compared with C_3 products, in triggering NCBP release.

Method Sera depleted of C_5 by exposure to the anti- C_5 gel, whether by incubation or on a column, were then activated with zymosan in the presence of 0.5M EACA and added to cytochalasin B treated PMN's as before. Since sera depleted by either the incubation or column methods gave similar results in the degranulation system, the data from the two methods have been pooled.

Congenitally C_5 deficient serum was also used in this experiment (kindly supplied by Dr R A Thompson). This was investigated at East Birmingham Hospital and found to be deficient in C_5 , while having normal levels of other complement components. Insufficient material was available to confirm these findings.

Results NCBP release in response to sera which had been C_5 depleted prior to zymosan activation was very markedly reduced compared to normal (Fig 20), and indeed at concentrations which would normally give more than 80% NCBP release, less than 5% release was obtained. These differences were highly significant statistically (Student's t test for paired samples). A similar reduction in NCBP release was seen when the low molecular weight fraction of serum which was C_5 depleted then zymosan activated in the presence of EACA was

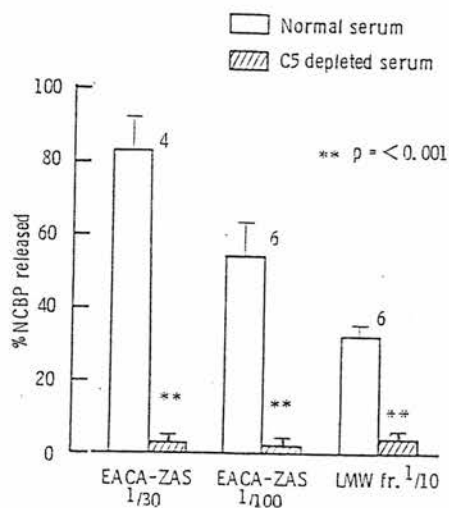


Fig 20. NCBP release by serum depleted of C₅ then zymosan activated, and by the low molecular weight fraction of that serum.

(mean ± SEM, n indicated above each pair).

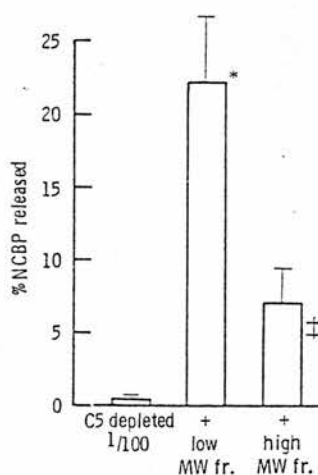


Fig 21. NCBP release from cytochalasin B treated PMN's exposed to C₅ depleted serum (1%) which was then zymosan activated. Restoration of degranulating activity of C₅ depleted serum by addition of fractions of EACA-ZAS.

* Addition of low MW fr produced significantly more NCBP release than C₅ depleted serum alone (p = 0.05, n = 4).

† Addition of high MW fr produced no significant increase in degranulation.

used (Fig 20).

Attempts were made to restore the degranulating activity of C_5 depleted serum by addition of the low molecular weight fraction (ultrafiltrate) or high molecular weight fraction (retained by the 30,000 Dalton exclusion filter) prepared from normal EACA-ZAS (Fig 21). There was a slight, but not significant restoration of activity by high MW fr, but a much greater and significant rise in degranulating activity was achieved by the addition of low MW fr, as expected from the results of experiment 7 above.

Activation of the congenitally C_5 deficient serum with zymosan, in the presence of EACA, yielded practically no degranulating activity, NCBP release being little greater than that seen with PBS-A alone (Fig 22). There was insufficient serum to prepare a low molecular weight fraction.

Experiment 11 NCBP release from PMN's by sera which had been zymosan activated, then exposed to the anti- C_5 gel.

The purpose of this experiment was to see whether the gel could remove C_5 -dependent degranulating activity, as well as unconverted C_5 .

Methods Both EACA-ZAS and its low MW fraction were treated with the anti- C_5 gel, either by incubation or on a column, then added to cytochalasin B treated PMN's as before.

Results Results by either method were identical and so the data were pooled. As shown in Figure 23, treatment of either EACA-ZAS or its low MW fraction produced no loss of degranulating activity when compared with untreated preparations. Thus, although the anti- C_5 gel was extremely

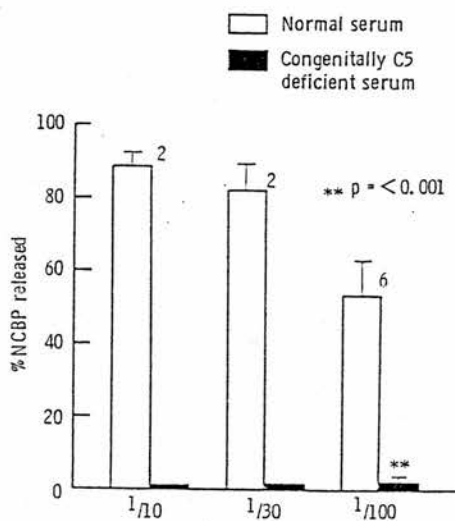


Fig 22. NCBP release from cytochalasin B treated PMN's by dilutions of EACA ZAS prepared from either normal serum or congenitally C₅ deficient serum.

(mean ± SEM, n indicated above each pair).

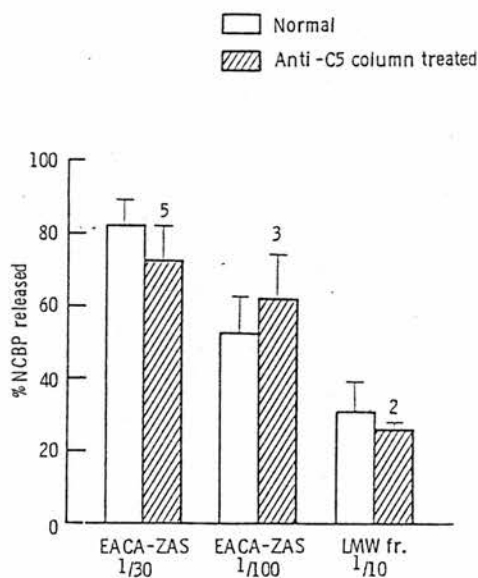


Fig 23. NCBP release by EACA-ZAS (or its low molecular weight fraction) produced by zymosan activation followed by passage over the anti-C₅ column (Mean ± SEM, n indicated above each pair).

effective at removing C_5 , it appeared not to bind C_5 -dependent degranulating activity.

Experiment 12 Use of antibodies in the degranulation system.

From the above data on C_5 deficient or depleted sera, it appeared that the major degranulating stimulus in ZAS or EACA was of low molecular weight and was derived from C_5 . It seemed likely that this stimulus was C_5 fragments, either C_{5a} or C_{5a} des Arg. To further test this possibility, and to specifically examine the role of C_3 conversion products in NCBP release, antisera to various serum components were mixed with either ZAS or low MW fraction before addition to the cells. As specific antisera to C_{3a}/C_{3a} des Arg and C_{5a}/C_{5a} des Arg were not readily available, antisera to whole C_3 and C_5 were used.

Method Initially 100 μ l EACA-ZAS and 100 μ l antibody were incubated together for 30 minutes at 37°C prior to addition to cytochalasin B treated cells. In later experiments, antisera were similarly incubated with the low molecular weight fraction of EACA-ZAS.

The following polyclonal antibodies were used (see Appendix 1 for details):

With ZAS : goat anti-human IgG
 : 2 different rabbit anti-human C_3 antisera
 : goat anti-human C_5

With LMW fr: 4 different anti-human C_3 antisera
 (2 rabbit, 1 goat, 1 sheep)
 : 2 different anti-human C_5 antisera
 (1 goat, 1 sheep)

Results When whole ZAS was the stimulus, anti-human IgG and 2

different anti- C_3 preparations did not cause any inhibition of degranulation (Fig 24). The addition of an anti- C_5 antiserum, however, caused marked inhibition of degranulation. This effect was even more striking when low MW fraction was the stimulus, for 2 different anti- C_5 antisera then caused complete inhibition of release (Fig 25). Again antisera to non-complement components cause no inhibition. Although 3 anti-human C_3 antisera were likewise non-inhibitory, partial (35%) release was seen with a fourth, an anti- C_{3d} preparation ($C_3(2)$ on Fig 25). This antiserum has been shown by Dr R J Powell to also react immunochemically with human C_5 , unlike those anti- C_3 preparations which did not inhibit NCBP release.

When the anti- C_5 antisera were diluted before addition to the degranulating system, their inhibitory effects could be overcome (Fig 26). Antisera differed in the concentration at which they ceased to be inhibitory, suggesting that they varied in their degree of activity against C_5 -dependent degranulating activity.

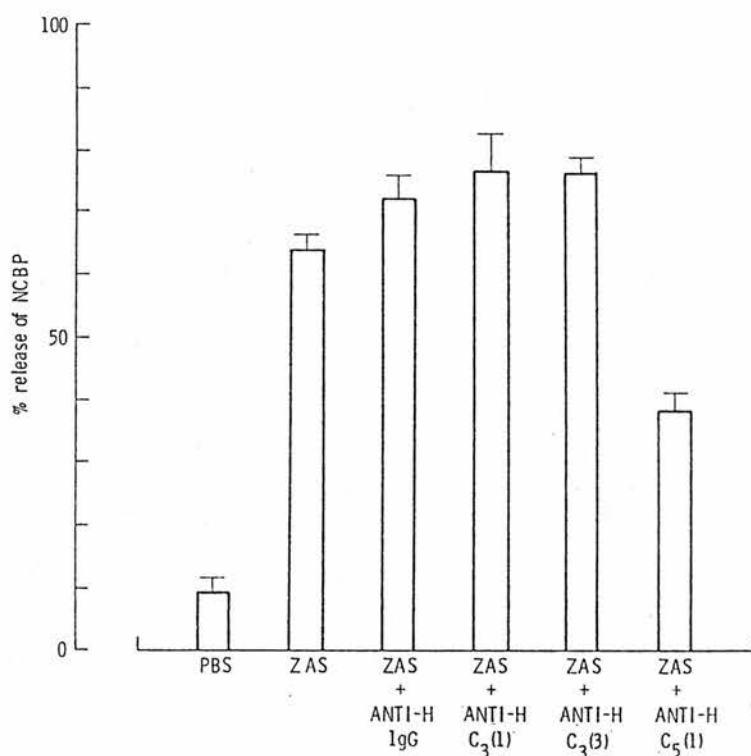


Fig 24. Effect of antisera to human IgG, C₃ and C₅ on NCBP release from human cytochalasin B treated PMN's by EACA-ZAS (Mean ± SEM, n = 4).

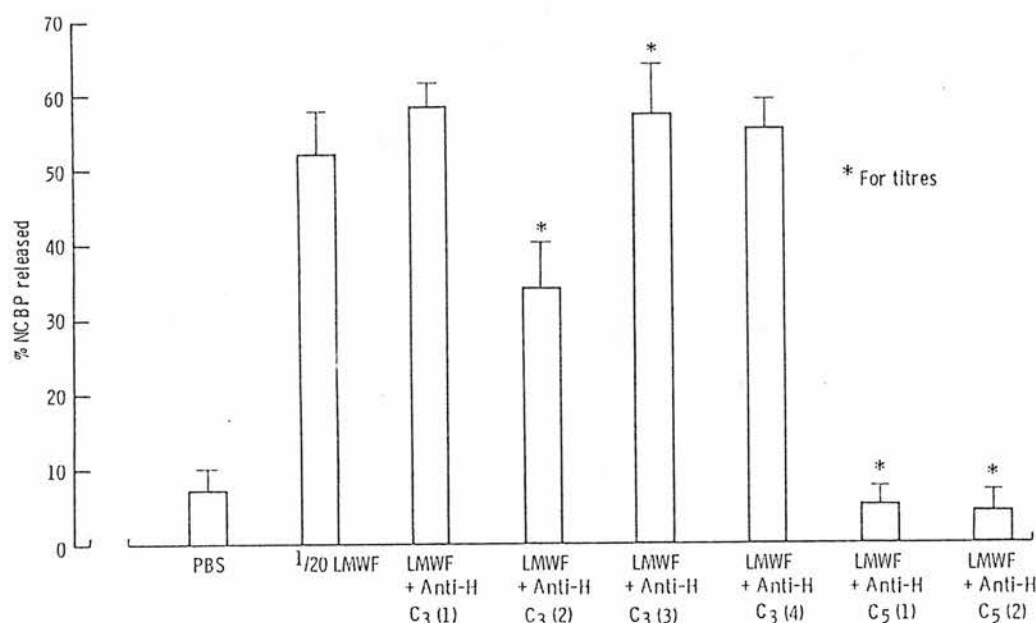


Fig 25. Effect of four antisera to human C₃ and two different antisera to C₅ on release of NCBP from cytochalasin B treated PMN's by the low molecular weight fraction of EACA-ZAS (mean ± SEM, n = 4).

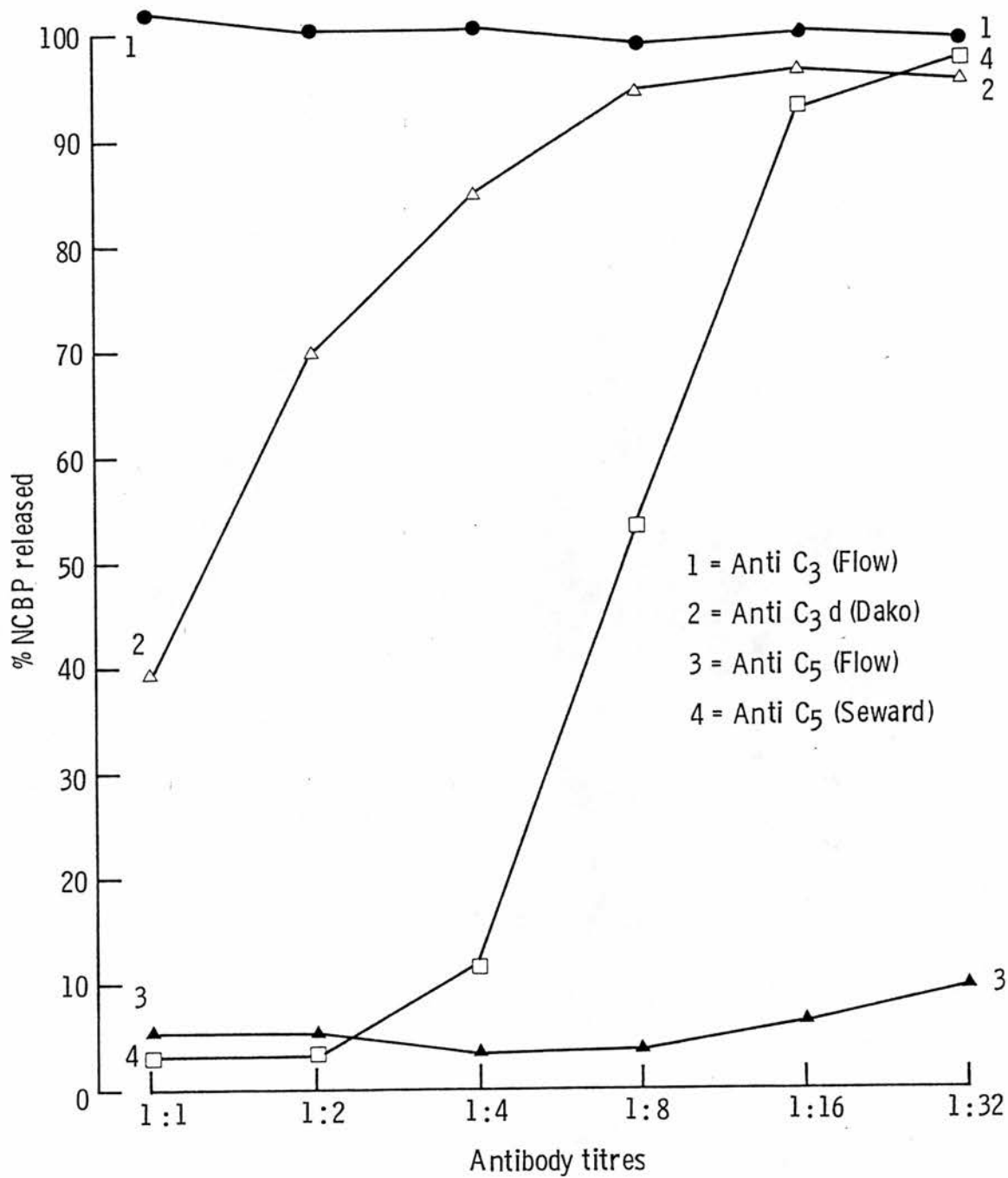


Fig 26. The effect of dilutions of anti-C₃, anti-C₃d and anti-C₅ antisera on NCBP release from cytochalasin B treated PMN's by low molecular weight fraction of EACA-ZAS.

Each result was expressed as a percentage of NCBP release obtained without antisera.

DISCUSSION

The work in this chapter reports dose-dependent release of the PMN secondary granule component NCBP, in response to a stimulus derived from the alternative complement pathway, as shown by the effects of heating and divalent cation chelators on the generation of degranulating activity.

The findings using column chromatography and the ultrafiltration membrane suggest that low molecular weight conversion products of either C_3 or C_5 are responsible. The absence of NCBP release with activated C_5 -deficient or C_5 -depleted sera (using an affinity gel which appears to remove only C_5) is consistent with the hypothesis that the main degranulating stimulus in either ZAS or EACA-ZAS is derived from C_5 . C_3 remains readily detectable in sera after exposure to the anti- C_5 gel, and will thus form C_{3a} and C_{3a} des Arg on zymosan activation. The fact that C_5 -depleted serum is totally inactive suggests that C_3 conversion products are not degranulating stimuli for PMN's, just as they have been shown not to be chemotactic⁶⁶. The data obtained using the gel are supported by the findings with congenitally C_5 deficient serum. It is not possible to study the effect of removal of C_3 on the generation of degranulating activity, since this would block the complement pathway totally, and prevent activation and conversion of C_5 and other later components. Although there is no absolute proof in these studies that the active moiety is actually C_{5a} and/or C_{5a} des Arg, the body of evidence in the literature from studies using pure compounds, suggests strongly that C_{5a} and C_{5a} des Arg are the main degranulating stimuli in

this system. Since degranulating activity is generated whether the carboxypeptidase B inhibitor EACA is present or not, it would appear that both C_{5a} and C_{5a} des Arg are effective, as would be expected from other studies on their chemotactic and degranulating properties^{22, 60}. EACA-ZAS, however, is only 5-10 times more potent than ZAS, whereas the ED_{50} 's for pure C_{5a} and C_{5a} des Arg with regard to PMN activation would predict that ZAS formed in the presence of EACA would be 100-1000 times more potent²². The apparent enhancement of effect of ZAS without EACA may lie in the fact that the activity of C_{5a} des Arg generated in whole serum may be enhanced by a high molecular weight 'helper factor' protein⁶³.

Some apparent anomalies in the results require consideration. Firstly, the activity of the low MW fraction of EACA-ZAS appears much less than that of unfractionated material, causing a maximum of 40-50% release of total NCBP, as apposed to the 80% or so maximal release triggered by whole EACA-ZAS. This loss of activity by fractionation was seen in several different batches of EACA and is probably explained simply on the basis of 'clogging up' of the ultrafilter with large proteins so that a proportion of serum, with solutes and low molecular weight peptides cannot pass through. Concentration of the ultrafiltrate by dialysis or lyophilisation would probably have yielded a more potent material. Secondly, it proved impossible to generate an active low MW fraction from ZAS formed in the absence of EACA. The material collected in the ultrafiltrate caused virtually no NCBP release. Although the reason for this is not absolutely clear, it may again be due to binding of C_{5a} des Arg to a high

molecular weight helper protein. Such binding would prevent C_{5a} des Arg from appearing in the ultrafiltrate, which would thus fail to trigger NCBP release.

Results of experiments using various antisera to detect, remove or inhibit either native C_5 or its conversion products have also produced some anomalies. For example, anti- C_5 antisera form visible precipitates with C_5 in unactivated and activated sera using IEP or immunodiffusion techniques, and, when coupled to a gel, effectively remove C_5 from whole unactivated sera. C_5 conversion products, however, are not detected by anti- C_5 by either IEP or immunodiffusion. Similarly, degranulating activity, which cannot be generated in congenitally C_5 deficient or C_5 depleted serum, and which therefore is likely to be derived from C_5 , is not removed by the anti- C_5 gel. This may be explained by the fact that the anti- C_5 antisera used were polyclonal antisera raised in animals, so that they would contain antibodies against many different antigenic determinants on the C_5 molecule. Little antibody may therefore have been formed specifically against the C_{5a} part of the molecule, which is only a small part of the end of the chain of C_5 . Thus adequate binding of C_{5a} would not necessarily be achieved. In addition, when the antiserum is exposed to whole ZAS or EACA-ZAS, it might preferentially bind to any unconverted C_5 , reducing the concentration of antibody available to bind C_5 fragments. This factor cannot however totally explain the inability of anti- C_5 to complex C_5 fragments, since low MW fraction, containing no C_5 , can be neither detected nor removed by anti- C_5 .

Given the inability of anti- C_5 to either detect or remove

C_5 fragments it is surprising that anti- C_5 , when incubated in the fluid phase with either ZAS or low MW fraction can block their ability to trigger NCBP release. This blockage is partial in the case of ZAS, again perhaps because of preferential binding to unconverted C_5 , but is total in the case of low MW fraction. This ability of various anti- C_5 antisera in the fluid phase to block the cellular effects of C_5 fragments is unlikely to be due to a direct effect of antibody on the cells, since control antisera had no such inhibitory effects on NCBP release. One possible explanation is that fluid phase incubation of antigens and antibody together allows more effective complexing to take place than during IEP or using the anti- C_5 gel. This would have to take place rapidly, to prevent the initiation of cellular events which normally follows occupation of receptors. Because C_{5a} and C_{5a} des Arg differ by only one arginine residue, it seems unlikely that anti- C_5 antisera would bind to one and not the other.

The apparent specificity of C_5 fragments in triggering NCBP release, and the difficulty in detecting these fragments immunologically, suggests that this degranulating system might be of value as a functional assay for complement activation in general, and C_5 conversion in particular. The most widely used assay to detect complement activation in present use is measurement of C_3 split products, particularly C_{3d} by IEP. This 2-stage procedure is time consuming and labour-intensive, and results are rarely available the same day. A double-decker rocket IEP technique has been described, however, which may shorten the procedure⁷⁴. Because of the unique actions of C_5 fragments on PMN's, however, and because such actions may

mediate tissue damage in disease states such as ARDS^{80, 81}, an assay for C_5 conversion may be a better predictor of outcome in these situations. This has been described in one study of ARDS⁹².

Previous functional assays for C_5 conversion products such as contraction of ileal smooth muscle depend on the anaphylotoxic properties of the C_{5a} molecule. They are therefore of very limited value in a clinical context, since C_{5a} des Arg, the stable form of C_{5a} in plasma, has only 0.1% the spasmogenic activity of C_{5a} , and is virtually undetectable by these means¹³⁹. Functional assays using PMN's have the advantage that both C_{5a} and C_{5a} des Arg can be detected, since both trigger chemotaxis, aggregation, and degranulation. Chemotactic systems do not readily lend themselves to use as an assay for C_5 conversion, since not many samples could be handled at once. In addition, depending on the system, C_{5a} des Arg may need the presence of the serum 'helper factor'⁶³. PMN aggregation has been used as a functional assay for C_5 fragments, and appears to be a more sensitive index of complement activation than either C_3 breakdown products detected immunochemically⁹² or C_5 fragments detected by chemotaxis⁷⁵. They do, however, require a platelet aggregometer, channeliser, and expertise in their use.

Degranulation assays, on the other hand, are easy to learn, are robust and reproducible, and require no special equipment. When neutrophils are stimulated by C_{5a} and C_{5a} des Arg to degranulate, primary granule markers such as B glucuronidase and myeloperoxidase can also be released⁶⁷, and could theoretically be used as the detection system. At the

same time, however, the superoxide anion is generated via the neutrophil oxidase²². Under such circumstances, β glucuronidase is liable to intracellular oxidative damage, resulting in up to 20% loss of detectable activity¹⁴⁰. The same problem arises with myeloperoxidase, where phagocytosis of opsonised zymosan by human neutrophils produces a 40-50% reduction in intracellular myeloperoxidase levels, while only 5-10% is detectable extracellularly¹⁴¹. In addition any functional assay system for myeloperoxidase will measure both neutrophil and eosinophil peroxidase. As eosinophil peroxidase is several times more potent than neutrophil peroxidase¹⁴², minimal amounts resulting from eosinophil contamination of neutrophil suspensions markedly influences results¹⁴³.

An alternative neutrophil enzyme to assay as a reflection of degranulation is lysozyme, which, although present in both primary and secondary granules, is released in similar proportions to NCBP¹⁴⁴. The principal advantage of NCBP over lysozyme in this system is that fewer cells are required (2×10^6 compared with 1×10^7), a factor which becomes important when large numbers of samples are to be tested. In addition, lysozyme, like myeloperoxidase and β glucuronidase, is liable to intracellular oxidation¹⁴⁰ and is more time consuming to assay than NCBP. The latter, however, is more expensive and requires the use of a radio-isotope label as well as a system free of extraneous cobalamin and cobalamin binding protein. Because of the presence of unsaturated cobalamin binding protein activity in human plasma, whole blood cannot be used as a "substrate" for test samples, as there would be competition between neutrophil CBP and the plasma binder for uptake of the

radiolabelled cyanocobalamin used in the assay. Availability of a one-stage neutrophil preparation medium¹³⁰, however, greatly shortens the duration of the assay, allowing its potential development in clinical situations.

Non-functional assays of C_5 conversion products are becoming available, using radioimmunoassay techniques^{145, 146}. These assays incorporate antisera specifically raised against C_{5a} or C_{5a} des Arg, and estimate their concentrations by competition with a known amount of radiolabelled C_{5a} for the antibody. However, it is recognized that even with an antiserum raised against C_{5a} , binding to whole C_5 may occur, giving falsely high results. The success of the assay depends therefore on initial removal of whole C_5 from the sample by precipitation with acid or other agents. The success of this awaits full evaluation, although the assays appear to be able to detect concentrations of C_{5a} 10-20 ng/ml¹⁴⁵, equivalent to $1-2 \times 10^{-6}$ M. This is greater than the estimated concentration of C_{5a} achievable in maximally activated plasma *in vitro*, although *in vivo* levels could be higher in states of rapid turnover. Comparison of NCBP release and radioimmunoassay as detection methods for C_5 conversion has not yet been made. One potential problem with immunological assays is the albeit rare situation of an immunochemically reactive but functionally defective molecule. This has been described in a case of congenital C_5 deficiency¹⁴⁷.

Whatever method is used for the detection of C_5 conversion, extreme care is needed in the method of blood collection and plasma separation. Artefactual complement activation can readily occur, for example if blood is allowed

to coagulate³, or by contact with siliconised plasma separators¹⁴⁸. An anticoagulant is therefore required, preferably in the syringe. However, this presents problems for the degranulation system described above. Firstly, heparin can itself occasionally activate PMN's, causing NCBP release from PMN's in the sample before the plasma can be separated³. The plasma will therefore contain spuriously high levels of NCBP which will be detected in the final assay system. This emphasises the importance of measuring the NCBP in all plasma samples tested, and subtracting the result from the total measured after PMN release. Other PMN factors contaminating plasma samples may further interfere with the assay by triggering complement activation *in vitro*, giving falsely high results. An alternative anticoagulant to use for blood collection would be EDTA, since this inhibits both coagulation and complement activation *in vitro*. However, this would interfere with degranulation by removing Ca^{++} and Mg^{++} from solution. Overcoming this by increasing the Ca^{++} and Mg^{++} concentrations then carries the risk of initiating coagulation. A suggested method of blood collection for C_5 conversion assay is therefore shown in Figure 27. This involves rapid removal of cells from plasma at 4°C before the addition of a small amount of heparin.

The two clinical situations in which measurement of C_5 conversion might be of most value are in ARDS and in joint inflammation. As discussed in Chapter 1, PMN activation by C_5 fragments is believed to play an important part in the lung damage seen in the former condition. Plasma from ARDS patients has been shown to contain PMN aggregating activity, likely to

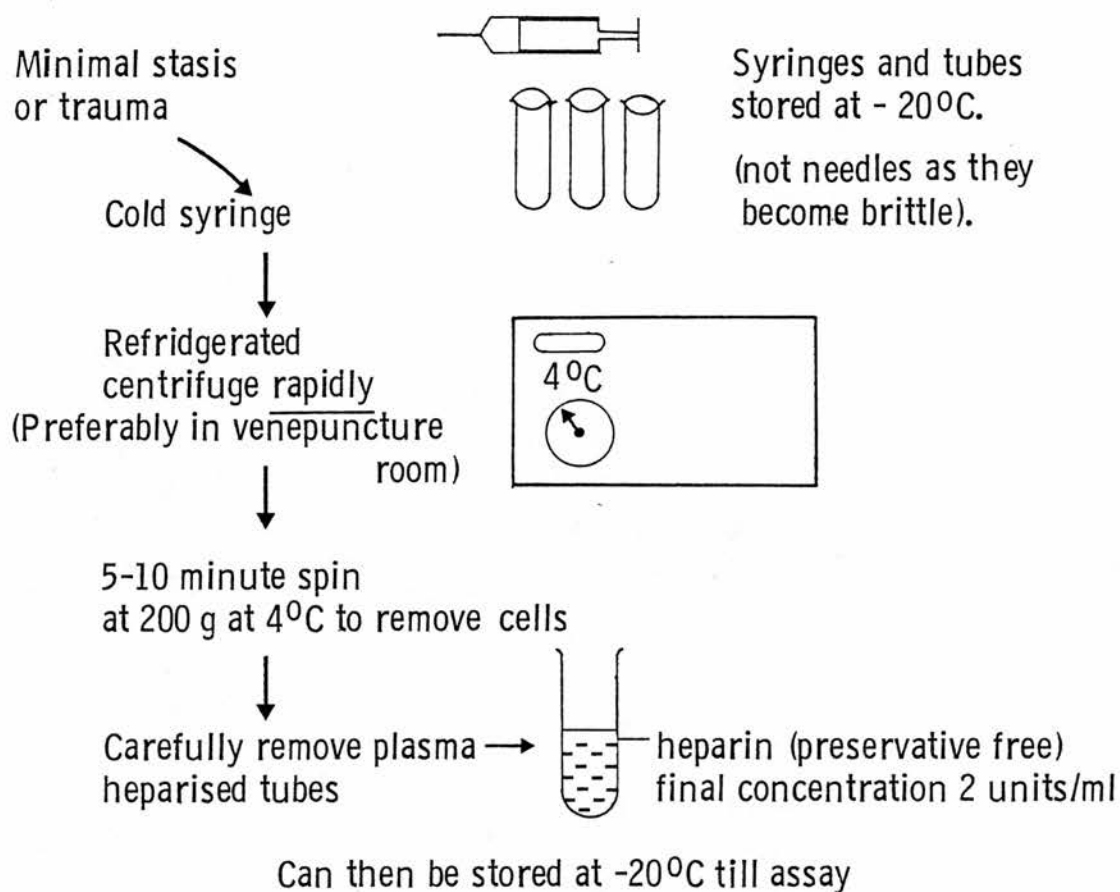


Fig 27. Suggested method of blood collection for assay of plasma C₅ fragments using PMN degranulation assay.

be C_{5a} des Arg⁹². This may be a more accurate predictor of ARDS than C_3 conversion and the facility of a rapid semi-quantitative assay for C_{5a} des Arg should now allow further prospective studies to be done, not only to monitor the progress of established ARDS, but as an early predictor for high risk patients on intensive care units. Activated complement components may be present in plasma before clinical features develop, and it is at this time, before lung damage is established, that therapeutic intervention should have its greatest chance of success. Examination of bronchial lavage fluid for C_5 fragments could also be performed.

Since activated components of the classical complement pathway have been found in synovial fluid in rheumatoid arthritis¹⁰² (see Chapter 1), examination of synovial fluid for C_5 fragments could aid in the diagnosis of the inflamed joint. In established rheumatoid disease, such examination would provide an opportunity to study local disease activity as it proceeds.

An assay of C_5 -related degranulating activity also has certain *in vitro* uses, and may be of value in quantitating the titre of anti- C_{5a} activity present in antisera. In experiments which examined the inhibition by antisera of NCBP release in response to activated complement, release was inhibited by two different anti- C_5 antisera but not by three different anti- C_3 antisera. The exception was the inhibition produced by a single anti- C_{3d} antiserum, but this antiserum also reacted immunochemically with human C_5 . To determine a titre of anti- C_{5a} activity, serial dilutions of test antisera could be incorporated into the degranulation assay.

A further application of this assay system is in the *in vitro* testing of anti-inflammatory agents. Indomethacin and other non-steroidal anti-inflammatory agents have been shown to inhibit both superoxide production⁴² and NCBP release⁹¹ from neutrophils triggered with soluble stimuli. In neither case was acetyl salicylic acid affective. Corticosteroids appear to inhibit both secondary⁹¹ and primary¹⁴⁹ granule discharge. The inhibitory effect of a given anti-inflammatory drug, however, does not correlate with its ability to block the cyclo-oxygenase pathway⁹¹ so that degranulation inhibition may provide a new model for assessing such agents. It may be particularly valuable in the development of agents active in the prevention of complement and neutrophil mediated damage as seen in ARDS and rheumatoid arthritis. Inhibition of NCBP release by pharmacological agents is considered further in chapters 6 and 7.

CHAPTER 3

CONDITIONS INFLUENCING COBALAMIN BINDING PROTEIN RELEASE FROM STIMULATED AND UNSTIMULATED NEUTROPHILS

INTRODUCTION

The work in the previous chapter was concerned with NCBP release from cytochalasin B (CB) treated PMN'S in response to C₅ derived fragments of activated complement. In this chapter, the following aspects of NCBP release will be considered:-

1. release by activated complement in non CB treated cells.
2. the kinetics of release in both CB and non CB treated cells (in collaboration with Dr Tony Brown).
3. release in non complement-activated serum, and the effect of cell concentration on this.
4. the role of the lipoxxygenase pathway of arachidonic acid metabolism in NCBP release (in collaboration with Dr Paul Maurice).

As mentioned in Chapter 1, CB greatly amplifies and speeds up PMN secondary granule release in response to various stimuli, but degranulation can occur in its absence. Comparison of the kinetics of NCBP release in the presence and absence of CB might indicate whether CB merely allows normal

secretory processes to take place more rapidly, or whether a different degranulation mechanism is induced.

When CB treated cells are exposed to serum in which complement system has not been activated, NCBP secretion is not significantly greater than in PBS-A alone³. This would suggest that no specific stimulus to NCBP release is present in unactivated serum. In the absence of CB, however, NCBP is slowly lost from PMN's suspended in such serum³. Moreover, PMN's from post-operative patients, which are refractory to *in vitro* NCBP release in response to activated complement also release much less NCBP than normal when suspended in unactivated serum³. Since NCBP release by such post operative PMN's is normal when FMLP is the stimulus, it is possible that unactivated serum also contains a specific degranulating stimulus to which post operative PMN's are desensitised. The nature of any such stimulus is not clear, but the complement system is one possible source. The role of C₅ activation in NCBP release by unactivated serum will therefore be examined, as will the effect of cell concentration.

Stimulation of PMN's by certain degranulation factors, such as the calcium ionophore A₂₃₁₈₇ also triggers metabolism of membrane arachidonic acid (AA)¹²⁵. One possible fate of AA is transformation via the enzyme 5'lipoxygenase, via a series of unstable intermediates, to the biologically active 5,12 dihydroxy-eicosatetraenoic acid, or leukotriene B₄ (LTB₄)¹²⁵ (Fig 28). LTB₄ can also trigger PMN reactions, including chemokinesis, and aggregation¹⁵⁰. It is therefore possible that LTB₄ acts as an intracellular messenger, and that its synthesis is a prerequisite for NCBP release in response to

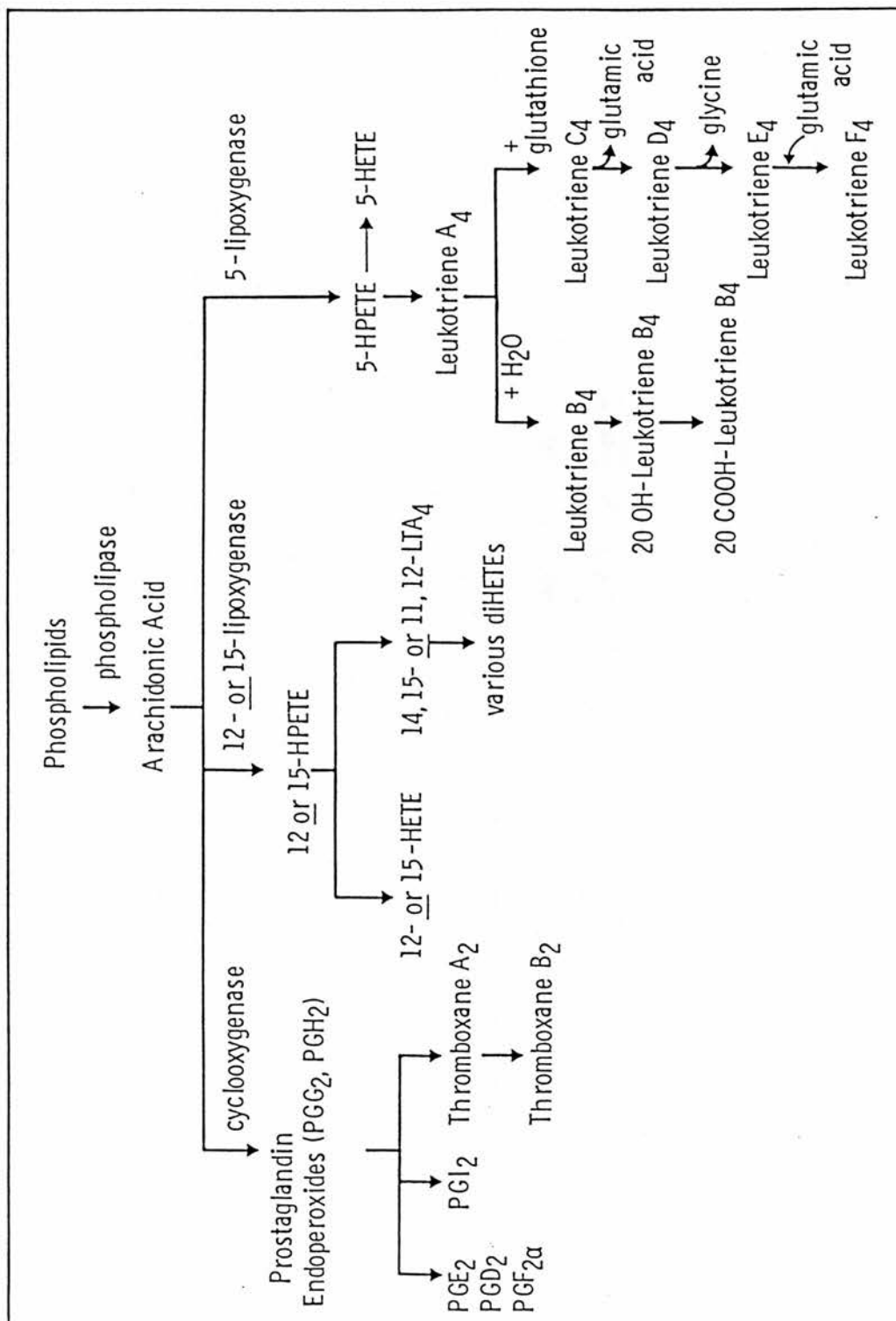


Fig 28. The pathways and products of arachidonic acid oxygenation.

stimuli such as C_5 fragments or FMLP. The literature on this is however conflicting, with some authors concluding that although LTB_4 synthesis and degranulation frequently occur together, they are independent events¹⁵¹. Others, however, believe that blockade of LTB_4 synthesis will prevent degranulation¹⁵². This point is therefore examined in this chapter, firstly by examining degranulation and lipoxygenase activity in parallel in response to FMLP and A_{23187} , and secondly by examining the effects of a known lipoxygenase inhibitor, BW755C, on NCBP release by these stimuli. Lipoxygenase products can be detected by providing a radiolabelled source of arachidonic acid, and separating the products by thin layer chromatography. The peaks of activity can then be counted and each product quantified.

EXPERIMENTAL WORK

Experiment 1 Stimulus-mediated NCBP release in cytochalasin

B and non-cytochalasin B treated cells.

Method The general method for the degranulation assay was followed. PMN's were incubated at 2×10^6 /ml in PBS-A with either unactivated pooled human serum, ZAS or C₅ depleted serum prepared as described in chapter 2, and incubated at 37°C for 45 minutes. The reaction was stopped by centrifugation at 4°C and NCBP assayed in the cell-free supernatants.

An additional stimulus used was formyl-methionyl-leucyl-phenylalanine (FMLP) dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO (<0.05%) was insufficient to interfere with either PMN responses or NCBP assay.

Result Pretreatment of PMN's with CB (5µg/ml) prior to exposure to either EACA-ZAS or LMW fr more than doubled the percentage of intracellular NCBP release (Fig 29), despite the fact that the incubation time with the stimulus was 5 minutes in the case of CB treated cells, and 45 minutes where CB was not used. Similar differences were seen when the stimulus was FMLP (Fig 30). The presence of CB did not increase release from cells suspended in PBS-A only.

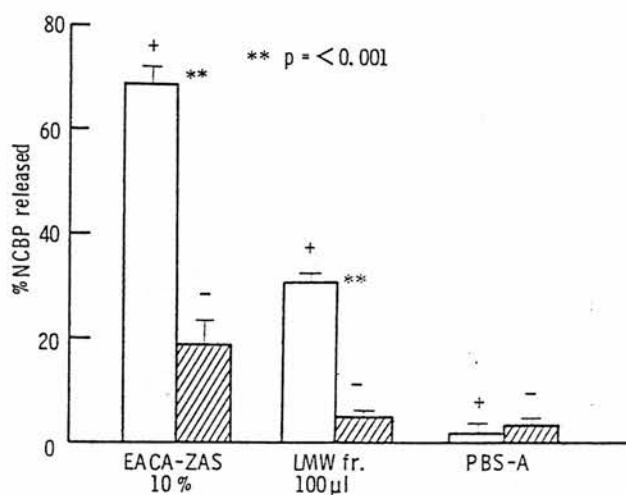


Fig 29. Release of NCBP by EACA-ZAS and its low MW fraction with (+) and without (-) cytochalasin B. (mean \pm SEM, n = 4).

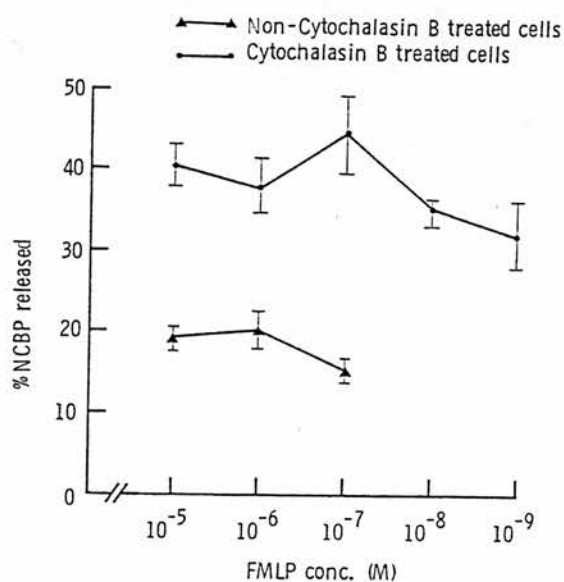


Fig 30. Release of NCBP by different concentrations of FMLP in the presence and absence of cytochalasin B. (mean \pm SEM, n = 4).

Experiment 2 Kinetics of NCBP release in response to FMLP

with and without cytochalasin B (in collaboration with Dr Tony Brown) .

A. With Cytochalasin B

Method A 20ml suspension of PMN's (2×10^6 /ml) in PBS-A was divided into 4 5ml aliquots and incubated for 5 minutes at 37°C with CB. FMLP (final concentration 10^{-5}M) or PBS-A was then added. Two of the 4 tubes were stirred - thus:-

- (i) - PBS not stirred
- (ii) - FMLP stirred
- (iii) - FMLP not stirred
- (iv) - PBS stirred

At each time point from 15-300 seconds, 0.4ml cell suspension was transferred to an Eppendorf tube and centrifuged for 10 seconds through dibutyl phthalate oil (to prevent diffusion of NCBP from cells into supernatant as they are spun) in a microcentrifuge. Replicates at 2 time points were centrifuged without oil to ensure that no NCBP was retained by the oil. The supernatants were removed for NCBP assay, and an aliquot of cells was sonicated to release all the NCBP as before.

B. Without cytochalasin B

Method PMN's (2×10^6 /ml) were incubated in PBS-A with FMLP 10^{-5}M , and the reaction stopped at time points from 10 seconds to 60 minutes by centrifugation as in (A) above.

Results There was no influence of stirring or of the presence of dibutyl phthalate oil on release or recovery of NCBP. In CB-treated cells, NCBP release proceeded extremely rapidly on addition of the stimulus (Fig 31), reaching 50% maximal

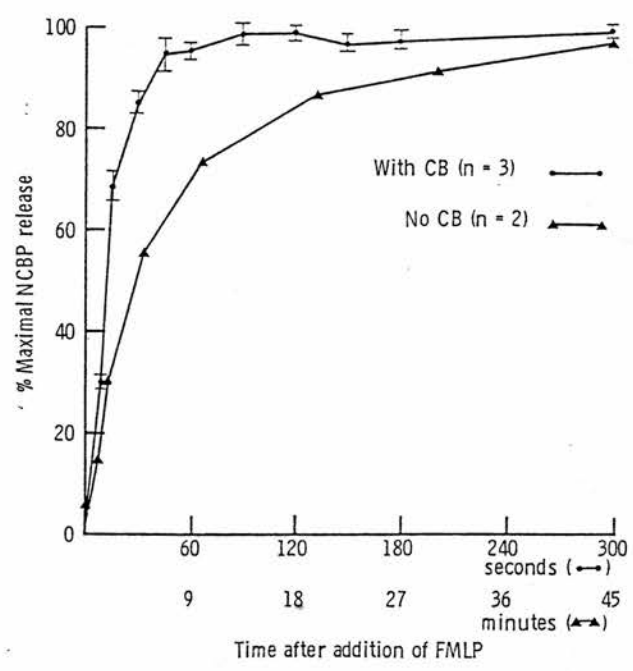


Fig 31. Kinetics of release of NCBP by FMLP 10^{-5} M with and without cytochalasin B.

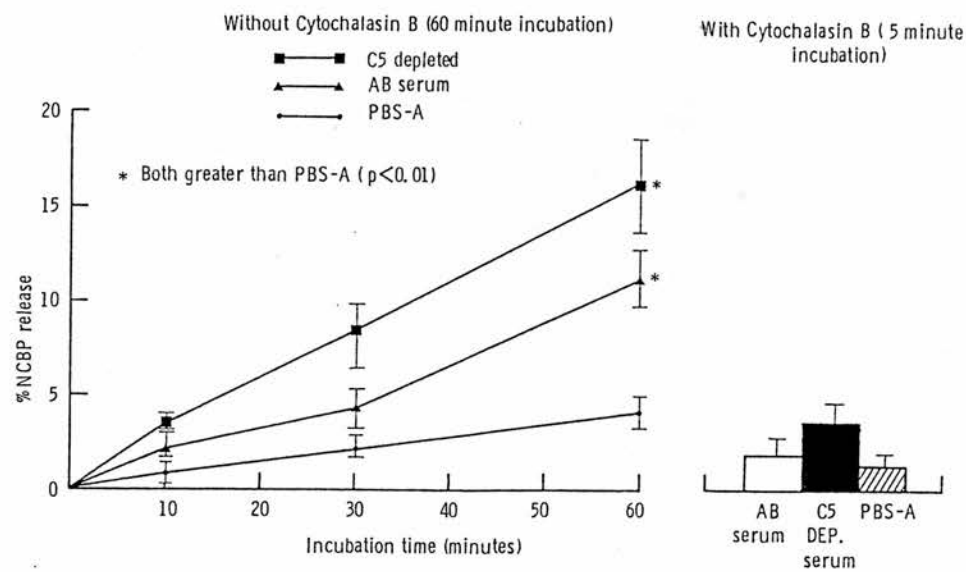


Fig 32. Release of CBP from PMN's in PBS-A, unactivated AB serum (5%) and unactivated C₅ depleted serum (5%) in the presence and absence of cytochalasin B.

release in 10-15 seconds, 95% maximal release in 60 seconds and a plateau of maximal release by 90 seconds. In contrast, non-CB treated cells took 5 minutes to reach 50% maximal release, and greater than 40 minutes to reach 95% maximal release. Considering that only 40% of the total intracellular NCBP was released during a 45 minute incubation with 10^{-5}M FMLP, it is likely that maximal release had not been reached within this time period, and that a slow increase in release would have persisted had the incubation been continued.

Experiment 3 Release of NCBP by non-cytochalasin B treated cells suspended in pooled unactivated AB serum and C_5 depleted unactivated serum.

Method A pool of unactivated serum was prepared from 2 normal subjects of blood group AB. This was chosen to minimise possible PMN activation due to red cell agglutination by anti-A or anti-B present in added serum. Non CB treated cells at various concentrations were incubated in this serum (final concentration 10%) for 45 minutes.

Some of the pool of AB serum was depleted of C_5 either by passage over the anti- C_5 column or by incubation with anti- C_5 gel as described in chapter 2. Non CB treated cells were incubated in this C_5 -depleted, non-activated serum for 45 minutes as above.

Results Cells incubated in PBS-A lost intracellular NCBP very slowly, with less than 5% secretion in 60 minutes (Fig 32). When AB serum was present, release was significantly greater, reaching 12% by 60 minutes. This release did not appear to depend on the presence of C_5 derived products, for release in

C_5 depleted serum was at least as great as, but not significantly greater than, that seen in AB serum. For a constant stimulus concentration, NCBP release in both ABS and C_5 -depleted serum was inversely proportional to the concentration of the cells over the range $4 \times 10^5 - 2 \times 10^7/\text{ml}$ (Fig 33). The effect of cell concentration was identical to that seen when either FMLP or EACA-ZAS was the stimulus (Fig 33).

Experiment 4 Measurement of lipoxxygenase products during NCBP release in response to FMLP and the calcium ionophore

A_{23187} (in collaboration with Dr Paul Maurice).

Method Venous blood was anticoagulated with EDTA (final concentration 5mM), and spun at 160g for 10 minutes to produce platelet-rich plasma, which was removed. Platelets have previously been shown not to affect NCBP release from PMN's³, but, since they also have a lipoxxygenase, they interfere with assay of lipoxxygenase products from PMN's. PMN's were then isolated by dextran sedimentation, removal of mononuclear cells by LSM, and hypotonic lysis of red cells as described in chapter 2. Aliquots of PMN's were suspended at $2 \times 10^6/\text{ml}$ in PBS-A (for degranulation studies) or at $1 \times 10^7/\text{ml}$ for leukotriene assay. Cytochalasin B, final concentration $5\mu\text{g}/\text{ml}$ was added, and the cells incubated for 5 minutes. The stimuli (A_{23187} $2\mu\text{M}$ or FMLP 10^{-5}M) and ^{14}C arachidonic acid in toluene ($60\text{mCi}/\text{mmol}$, final concentration $1.6\mu\text{M}$) were then added, and the incubation continued for a further 5 minutes. Control cells had unlabelled AA $1.6\mu\text{M}$ added. The reactions were stopped either by centrifugation at 4°C (for degranulation) or

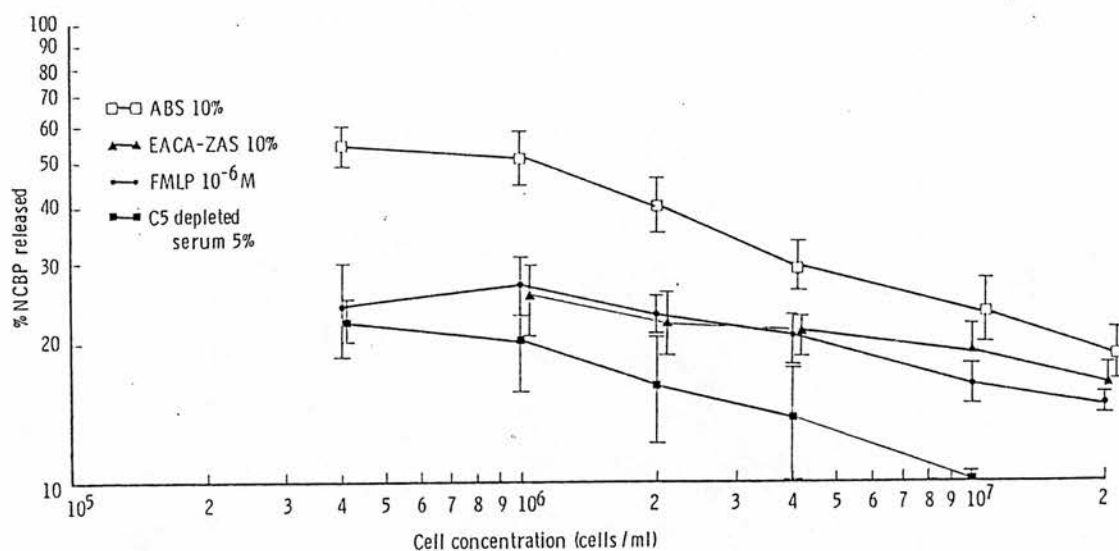


Fig 33. The effect of cell concentration on NCBP release in non cytochalasin B treated cells.

(mean \pm SEM, n = 4).

by acidification to pH 3 (for lipoxygenase products assay).

NCBP was assayed in cell-free supernatants as before.

Lipoxygenase products were detected by thin layer chromatography (TLC) (kindly performed by Dr Paul Maurice) as follows:-

1. The lipid products in the cell-free supernatants were extracted twice in ethyl acetate and the residue resuspended in a small volume of ethyl acetate.

2. The samples were applied to multi-lane TLC plates (Whatman LK6D).

3. The plates were developed in toluene : dioxane : acetic acid (65:34:1) at 4°C^{153} . This gives good separation of cyclooxygenase and lipoxygenase products.

4. The plates were scanned to locate peaks of radioactivity. These areas were scraped and counted in a liquid scintillation counter.

The results of each peak were expressed as a percentage of total radioactivity added. Arachidonic acid not converted into cyclooxygenase or lipoxygenase products is incorporated into neutral lipids.

Results When FMLP was the stimulus, very little arachidonic acid was metabolised to either the cyclooxygenase product PGE_2 or the lipoxygenase products 5 hydroxyeicosatetraenoic acid (5 HETE) or 5,12 diHETE (LTB_4) (Fig 34), despite nearly 80% NCBP release. In contrast, stimulation with A_{23187} , causing only 45% NCBP release, was accompanied by much greater lipoxygenase activity, with 20% of the labelled arachidonic acid appearing as LTB_4 (Fig 34).

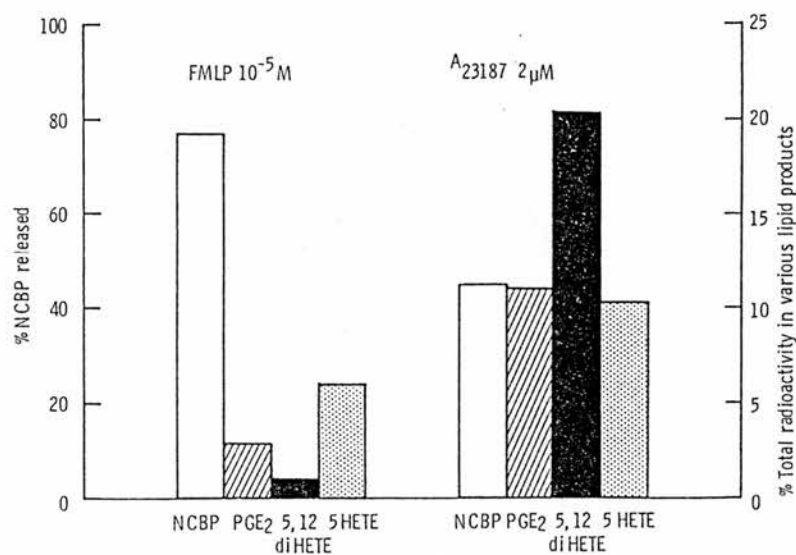


Fig 34. NCBP release and synthesis of PGE_2 , 5, 12 diHETE and 5 HETE in cytochalasin B treated PMN's by FMLP and A_{23187} . (n = 2)

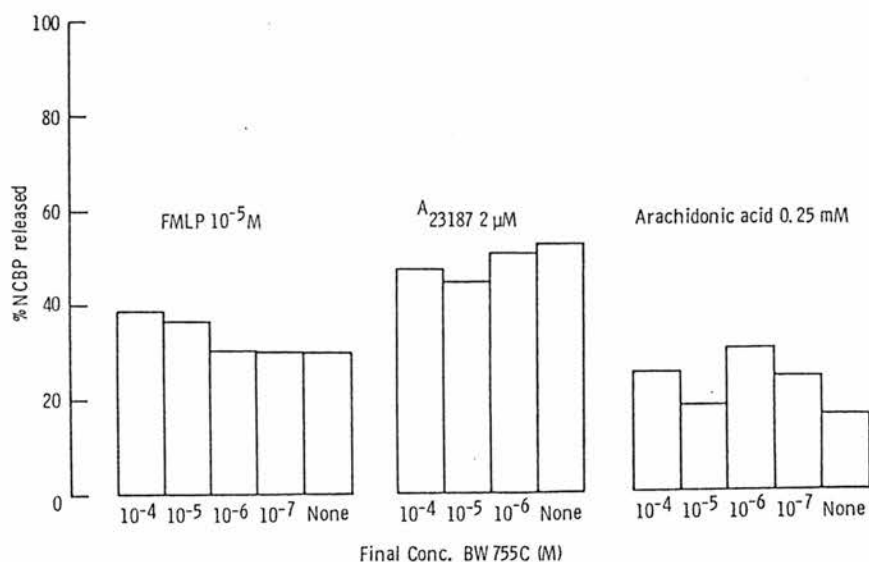


Fig 35. NCBP release from cytochalasin B-treated PMN's by FMLP, A_{23187} , and arachidonic acid: effect of the lipoxigenase inhibitor BW755C. (n = 2)

Experiment 5 Effect of BW755C on NCBP release in response to different stimuli

Method The lipoyxygenase inhibitor BW755C was dissolved in DMSO, and incubated at various concentrations with 2×10^6 PMN's/ml for 5 minutes. Because of the possibility of BW755C binding to albumin, none was added to the PBS, and non serum derived stimuli were used. Cytochalasin B (final concentration 5 μ g/ml) was then added and the incubation continued for a further 5 minutes. Three different degranulating stimuli were then used:-

- (i) FMLP (final concentration 10^{-5} M)
- (ii) the calcium ionophore A_{23187} in DMSO (final concentration 2 μ M).
- (iii) arachidonic acid in hexane (final concentration 0.25mM.)

After 5 minutes with the stimulus, the reaction was terminated by centrifugation at 4°C and NCBP assayed in the supernatants as before.

Results BW755C did not interfere with NCBP assay nor did it cause any NCBP release when added to the cells alone. At final concentrations up to 10^{-4} M, BW755C did not cause any inhibition of NCBP release by any of the stimuli examined (Fig 35). This contrasts markedly with its effect on lipoyxygenase product synthesis at lower concentrations (Table 7, courtesy of Dr Paul Maurice). While arachidonic acid alone at micromolar concentrations caused no stimulation of prostaglandin E_2 or LTB_4 synthesis (Table 7, column 1), addition of A_{23187} caused 35-40% of the radiolabel to appear in PGE_2 , LTB_4 or the LTB_4 precursor 5 HETE (column 2). The presence of BW755C with A_{23187} , however, completely blocked

Table 7

The effect of BW755C on arachidonic acid
metabolism by PMN's with and without A₂₃₁₈₇.

(% incorporation of recovered radioactivity into products :
each value is a mean of 2).

| | AA (4 μ M) only | AA + A ₂₃₁₈₇ (4 μ M) | AA+ BW755C (8 x 10 ⁻⁵ M) | AA+ A ₂₃₁₈₇ + BW755C (8 x 10 ⁻⁵ M) |
|-------------------------------------|-------------------------|--|---|---|
| Polar lipid | 7.3 | 14.4 | 6.2 | 38.4 |
| PGE ₂ | - | 4.2 | - | - |
| T x B ₂ | 3.0 | - | - | - |
| 5, 12 diHETE (LTB ₄) | - | 18.4 | - | - |
| 5 HETE | - | 14.4 | - | 1.1 |
| 12 HETE | 14.4 | 6.8 | 0.2 | - |
| AA | 2.6 | 3.1 | 3.8 | 8.3 |
| Neutral lipids | 52.7 | 23.8 | 86.3 | 44.2 |

AA = arachidonic acid

production of any of these metabolites, so that practically all the radiolabel appeared in either neutral or polar lipids.

DISCUSSION

As is well documented⁴⁹, preincubation of PMN's with CB both greatly amplifies and accelerates NCBP release in response to whole EACA-ZAS, its low molecular weight fraction, or FMLP, all of which trigger non-cytotoxic dose-dependent degranulation via surface receptors. The kinetic pattern, however, appears similar whether CB is present or not. There is initially rapid secretion of NCBP, slowing down with time, perhaps due to loss of surface receptors by internalisation of receptor-ligand complexes. The similarity in kinetics of release with and without CB would suggest that CB enhances normal stimulus-induced secretory processes, rather than triggering or permitting NCBP loss from the cells by other means.

The apparent anomaly in the results is that while release by unactivated AB serum is no greater than in PBS-A for CB-treated cells, release is much greater in AB serum than in PBS-A when no CB is present. Similarly, when no CB is present, cells secrete just as much NCBP in ABS as they do in ZAS, whereas in CB treated cells ZAS stimulates considerably more secretion. Release of NCBP in the presence of AB serum thus seems to differ fundamentally from release triggered by specific complement fragments. The degranulating activity in AB serum is stable at room temperature for 16 hours, and is only partially abrogated by heating to 56°C for 30 minutes, suggesting that it is not derived from complement³. Release from non-CB treated cells is also seen in C₅-depleted serum, and in plasma prepared by the 'rapid spin' technique designed to minimise complement activation³, suggesting that

serum-dependent release is not mediated via C_5 fragments. Chromatography of AB serum on Sephadex G200 reveals 1 peak of degranulating activity of high molecular weight for non-CB treated cells yet none for CB-treated cells³. A similar high MW peak appears to be present in ZAS, as well as the low MW peak representing C_5 fragments. The high MW peak, however, has no activity for CB treated cells. Release in ABS by non-CB treated cells varies with the concentration of both serum³ and cells, as shown above, in the same way as degranulation in response to known receptor-mediated stimuli such as EACA-ZAS and FMLP. The stimulus-specific loss of responsiveness of post operative PMN's to ABS and EACA-ZAS might also argue for a specific receptor-mediated mechanism. However, in normal cells in ABS concentrations as high as 60%, release is never greater than 25% of the total NCBP. Release also proceeds slowly in whole blood³. It would thus appear that 2 serum-derived degranulating stimuli can be identified. The first is of high molecular weight, and is present in both ZAS and ABS. It degranulates non CB treated cells slowly and its activity is not enhanced by CB. The second stimulus is of low molecular weight and arises from serum complement, probably from C_5 . It also degranulates non CB treated cells slowly but its activity is greatly enhanced by the addition of CB.

It has been suggested that PMN secondary granules may contain a labile subpopulation which are slowly lost from the cell under appropriate conditions, as when serum is present³. The presence or absence of CB would thus be irrelevant to such granule loss. The lack of secretion in CB treated cells could

be simply due to the much shorter incubation time used when CB is present, i.e. 5 minutes instead of 45 or 60 minutes. This slow 'background' release of secondary granules could influence various systems - granulopoiesis, via lactoferrin⁵⁷, reactivity of other PMN's via lysozyme⁵⁰, and possibly bacterial function via binding of cobalamins by NCBP. Thus NCBP release in normal serum may be a physiologically normal and desirable phenomenon, although its exact mechanism remains unclear.

NCBP release by CB-treated cells in response to FMLP is accompanied by very little synthesis of lipxygenase products as assayed by thin layer chromatography. This is consistent with previous observations using radioimmunoassay for LTB₄, in which the primary granule marker β glucuronidase was released from CB-treated cells by FMLP without detectable LTB₄ synthesis¹⁵¹. Receptor-mediated stimuli such as FMLP therefore appear much less able to stimulate leukotriene production than does A₂₃₁₈₇. The TLC method used in our study to detect lipxygenase products also detected other stable products of the lipxygenase pathway, and none was produced in significant quantities. The calcium ionophore A₂₃₁₈₇, however, triggered both NCBP release and synthesis of cyclooxygenase and lipxygenase products in significant quantities. The different behaviour of these stimuli may relate to the direct effects of the ionophore on cytoplasmic calcium levels, while FMLP acts via occupation of surface receptors, followed by calcium mobilisation and breakdown of membrane phosphotidyl lipids. In any case, it appears that NCBP release can occur without significant lipxygenase

activity. The behaviour of cells exposed to BW755C confirms this, with concentrations of drug shown to totally block lipooxygenase having no effect on NCBP release. This finding is similar to that of Palmer and Salmon¹⁵¹. In inflammatory sites, secondary granule release and leukotriene synthesis would both modify the inflammatory reaction. LTB_4 would recruit and activate further PMN's, while release of lysozyme could localise PMN's to inflammatory sites. Within individual cells, however, secondary granule release and leukotriene synthesis appear to be separate events.

CHAPTER 4

THE ROLE OF C₅ AND NEUTROPHILS IN THE WHEEL AND FLARE RESPONSE TO INTRA-DERMAL ZYMOSAN ACTIVATED SERUM (ZAS).

INTRODUCTION

Activation of PMN's by C₅ conversion products **in vitro** clearly triggers a rapid secretory response by the cells, as shown above, as well as chemotaxis and aggregation. C₅ products have also been implicated **in vivo** in the increased vascular permeability and inflammatory tissue damage seen in conditions such as the adult respiratory distress syndrome and rheumatoid arthritis, as discussed in Chapter 1. The question to be investigated in this chapter is whether PMN recruitment by C₅ fragments can be implicated in the immediate vascular response to activated complement in the skin.

Injection of activated complement into the skin produces an immediate wheal and flare response, but the exact mechanism remains unclear. The anaphylotoxins C_{3a} and C_{5a} cause release of histamine and leukotrienes from mast cells, but the des Arg form of C₅, with little activity towards mast cells, remains active in the skin, suggesting that a mast cell independent mechanism may be operating. The observations of Issekutz¹¹⁷ and Williams¹²¹ on the role of PMN's in the skin response to complement in the rabbit suggest that PMN products may mediate the permeability response. Their studies also throw into question the role of C₃ fragments in the skin reaction, since they have little chemotactic activity towards PMN's⁶⁶. The

role of C_5 fragments and neutrophils in the skin response to complement in human subjects will thus be examined here.

While studies with normal and neutropenic animals suggest that circulating PMN's are necessary for the vasodilatation and microvascular permeability response to intradermal C_{5a} ^{117,121} (reviewed in Chapter 1), no similar studies have yet been performed in neutropenic human subjects. There are obvious ethical and practical limitations to such work. The main one is the necessity to inject each subject's skin only with complement products from his or her own serum, rather than with a well characterised and standardised batch of C_{5a} prepared from a serum pool. Another difficulty is in obtaining patients with severe enough neutropenia, yet who are well enough to be studied and totally infection-free. This has of necessity limited the number of neutropenic patients who could have been studied.

The other point which is examined here is whether Williams' "two-mediator hypothesis" of inflammation applies to this skin model in man. The hypothesis states that demonstrable inflammatory reactions require both a vasodilator and a permeability producing mediator¹²³ (discussed in Chapter 1). Thus C_{5a} alone, despite increasing permeability, would not produce a wheal, as no vasodilator component would be present. This was borne out in studies in the rabbit¹²¹, where wheals were not produced by C_{5a} unless PGE_2 has been present also. To see whether this two mediator hypothesis applies in man, the skin response to ZAS and to PGE_2 has been examined for synergy between the two mediators. Synergy between two mediators exists if the response to a mixture of

the two is greater than the sum of the responses when the two mediators are injected separately.

EXPERIMENTAL WORK

Experiment 1 Preliminary studies on technique

A. Assessment of accuracy of measurement of 50 μ l injection volume.

Method By weighing weigh boats on an analytical balance before and after the addition of 50 μ l distilled water from a 1 ml syringe, the accuracy and reproducibility of this method of measuring 50 μ l could be assessed.

Results The results are shown in Table 8. The mean weight of 10 50 μ l aliquots was 50 mg weight and all values lay within 10% of that figure, which was considered acceptable for this study.

B. Reproducibility of measurement of wheal volume.

Method To assess the reproducibility of wheal response in different subjects, and within each subject at different times, repeat injections of dilutions of ZAS were made over several weeks, and the wheal volume measured as described below. Any variability in the results would therefore reflect true variability of response, as well as inconsistencies in measurement.

Results The results are shown in Table 9. The reproducibility of wheal volumes at 12 minutes by both concentrations of ZAS was acceptable considering the number of variables involved (see discussion). There was, however considerable inter-subject variation in response. By 30 minutes, both intra- and inter- subject variation was much greater than at 12 minutes. Therefore, although many experiments have measurements taken at both 12 and 30 minutes, the 12 minute measurements are probably more meaningful.

Table 8

The weights of 10 50 μ l aliquots of distilled water

| No | Weight (mg) | No | Weight (mg) |
|-----------------|-------------|-----------------------------------|-------------|
| 1 | 46.5 | 6 | 51.0 |
| 2 | 50.8 | 7 | 44.3 |
| 3 | 54.0 | 8 | 52.3 |
| 4 | 52.6 | 9 | 47.8 |
| 5 | 47.7 | 10 | 52.8 |
| Mean = 50.00 mg | | Standard error of the mean = 1.02 | |

Table 10

Comparison of wheal volumes produced by buffered and non buffered saline (n = 6)

| Time (mins) | Wheal volume (mm ³) | |
|-------------|---------------------------------|---------------------------|
| | Unbuffered saline | Phosphate buffered saline |
| 6 | 12.11 | 15.67 |
| 12 | 8.88 | 8.94 |
| 20 | 3.76 | 2.78 |
| 30 | 0.00 | 0.00 |

Table 9

Wheal volumes at 12 and 30 minutes after injections of ZAS : assessment of reproducibility. Results 1 and 2 were obtained from injection of the same material days or weeks apart.

| Subject | Injection | | Injection | |
|---------|-----------------------------|--------|-----------------------------|--------|
| | ZAS $\frac{1}{2}$: 12 mins | | ZAS $\frac{1}{2}$: 30 mins | |
| | Result 1 | 2 | Result 1 | 2 |
| 1 | 105.67 | 100.37 | 96.09 | 93.01 |
| 2 | 107.35 | 86.05 | 121.66 | 114.66 |
| 3 | 126.08 | 128.07 | 204.92 | 158.76 |
| 4 | 90.49 | 99.70 | 38.59 | 58.62 |
| 5 | 83.23 | 101.46 | 115.68 | 161.63 |
| 6 | 132.38 | 131.67 | 86.60 | 124.99 |
| 7 | 79.77 | 69.39 | 86.47 | 43.02 |
| 8 | 70.12 | 99.39 | 50.32 | 77.99 |
| 9 | 76.57 | 59.17 | 62.34 | 39.27 |

| Subject | Injection | | Injection | |
|---------|-----------------------------|-------|-----------------------------|-------|
| | ZAS $\frac{1}{2}$: 12 mins | | ZAS $\frac{1}{2}$: 30 mins | |
| | Result 1 | 2 | Result 1 | 2 |
| 1 | 63.40 | 84.30 | 17.85 | 11.82 |
| 2 | 68.70 | 86.65 | 106.89 | 42.18 |
| 3 | 52.08 | 55.73 | 74.93 | 74.68 |
| 4 | 62.59 | 44.31 | 59.15 | 41.25 |
| 5 | 43.30 | 33.58 | 16.37 | 10.44 |

C. Comparison of wheals produced by sterile, unbuffered 0.9% NaCl and phosphate buffered saline (pH = 7.4).

Sterile, pyrogen tested saline has no buffering capacity, but phosphate buffered saline made from laboratory chemicals, while it can be autoclaved, is not necessarily pyrogen free. A comparison was therefore made of wheals produced by each of them (Table 10), which shows no significant differences. Sterile pyrogen-tested saline was therefore used for all subsequent dilutions and injections.

Experiment 2 Wheals and flares produced by ZAS preparations in normal subjects.

Normal subjects were informed medical and laboratory staff of both sexes aged 21-49.

Methods

A. Preparation of activated complement.

Serum from each subject was incubated with boiled washed zymosan 2 mg/ml at 37°C for 60 minutes. Complement activation was terminated by centrifugation at 2000 g for 5 minutes followed by passage through a 0.2 μ filter. A partially purified low molecular weight fraction was prepared from zymosan-activated serum (ZAS) which was layered over a 30,000 Dalton micro-partition membrane and centrifuged at 2000 g for 30 minutes at room temperature. The fraction of ZAS which had passed through the membrane was used for injection. All manoeuvres were performed under sterile conditions in a laminar flow cabinet. Sera were stored under liquid nitrogen and were sterilised by filtration immediately prior to injection.

B. Injection of solutions.

Control solutions injected were saline, the supernatant from zymosan incubated alone, and serum not exposed to zymosan. Solutions were coded by someone not involved in the measurement and were injected randomly at numbered sites on the flexor surfaces of both forearms, which had been cleaned with alcohol. 50 μ l of each solution was injected from a 1 ml syringe through a 27 gauge needle into the skin so that a raised 'bleb' was formed on the skin surface (Fig 36). Where the solution entered subcutaneously, no such bleb was formed, and the injection was repeated at a fresh site.

C. Measurement of response.

Flare areas 10 minutes after injection, were calculated by measuring 2 diameters, 1 in the long axis of the arm (D_1) and 1 at right angles to it (D_2), and calculating the area from the formula

$$\text{Area (mm}^2\text{)} = \frac{\pi}{4} \times \frac{(D_1 + D_2)^2}{2}$$

where D_1 and D_2 are in mm.

Wheal volumes were calculated from wheal area, measured as for flare area, and wheal thickness. 'Pseudopodia' were not included in the measurement of wheal diameters. Wheal thickness was calculated by measuring a double thickness of skin (excluding sub-cutaneous tissue) at each site before and after injection, using spring-loaded skin calipers (Fig 37). This technique has previously been described¹⁵⁴ and validated¹⁵⁵. The calculation of thickness and volume is shown in Fig 38. Because the skin calipers measure 2 thicknesses of skin, the final thickness of the wheal is half



Fig 36. Intradermal injection of ZAS to produce a raised 'bleb' on the skin.



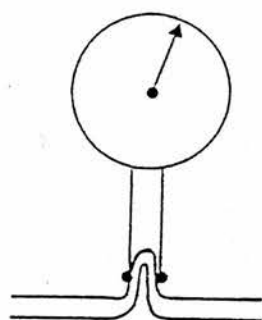
Fig 37. Measurement of wheal thickness using spring-loaded skin calipers.



Pre-injection

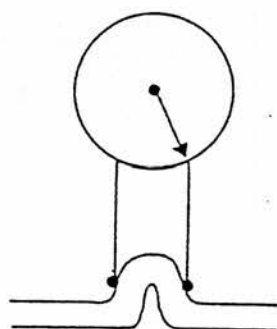
Wheal vol. \propto cylinder volume

$$= \frac{\pi d^2}{4} \times \text{height (wheal thickness)}$$



$$= \text{thickness} \times 2$$

Post-injection



$$= \text{thickness} \times 2$$

$$\text{Wheal volume (mm}^3\text{)} = \frac{\pi}{4} \times \text{mean diameter}^2 \times \frac{\Delta \text{thickness}}{2}$$

Fig 38. Technique and formula for measurement of skin wheals.

the difference in skin caliper measurement pre and post injection. To simplify the volume calculation, the formula for the volume of a cylinder is used, where wheal area = base and thickness = height. While the wheal is obviously not cylindrical, its volume will be proportional to that of a cylinder with the same base area and height. Each set of injections included a saline control, the response to which was subtracted from all other results when calculating volumes, unless otherwise stated.

Results Injection of 50 μ l sterile saline always resulted in a wheal volume at 12 minutes of less than 10 mm³ (Fig 39). No significant increase on this was produced by either unactivated serum or zymosan supernatant (Fig 39). Dilutions of ZAS, however, produced a dose dependent wheal and flare response by 12 minutes (Fig 40). The flare appeared within minutes of injection, followed by the wheal, which was readily measurable with well-defined edges by 12 minutes (Fig 41). At lower serum concentrations, the wheals were reduced in size by 40 minutes, although with neat serum an increase in wheal size occurred between 12 and 30 minutes (Fig 42). All wheals faded over an hour or two, and no late reactions were noted, but unlike histamine (see Chapter 5), injection of ZAS was not associated with any pain or itching. Injection of the low molecular weight fraction of ZAS also produced a wheal and flare response (Fig 43), although less than that produced by whole ZAS. Quantitative comparisons of potency with whole ZAS are not meaningful, because not all low molecular weight fraction is recoverable from the fractionation process. The fact that the low molecular fraction of ZAS can generate a

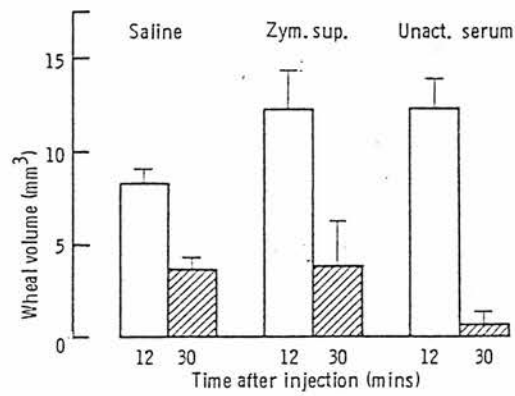


Fig 39. Wheal volumes in normal subjects 12 and 30 minutes after injection of saline, zymosan supernatant and unactivated serum. (mean \pm SEM, n = 10).

Not corrected for saline alone.

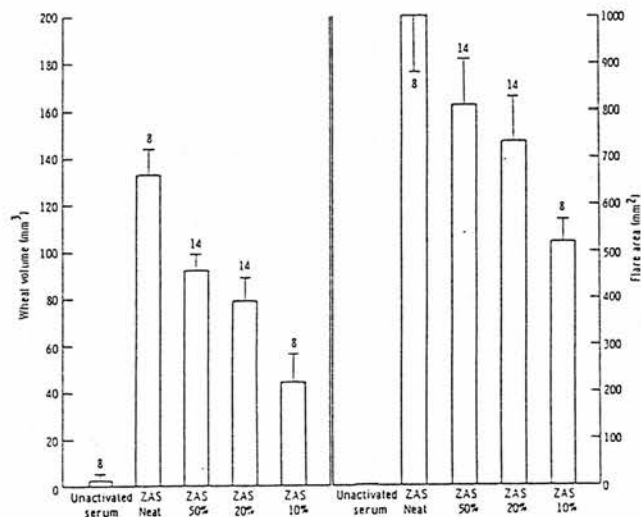


Fig 40. Wheal and flare responses to dilutions of ZAS at 12 minutes (mean \pm SEM, n shown above each bar).

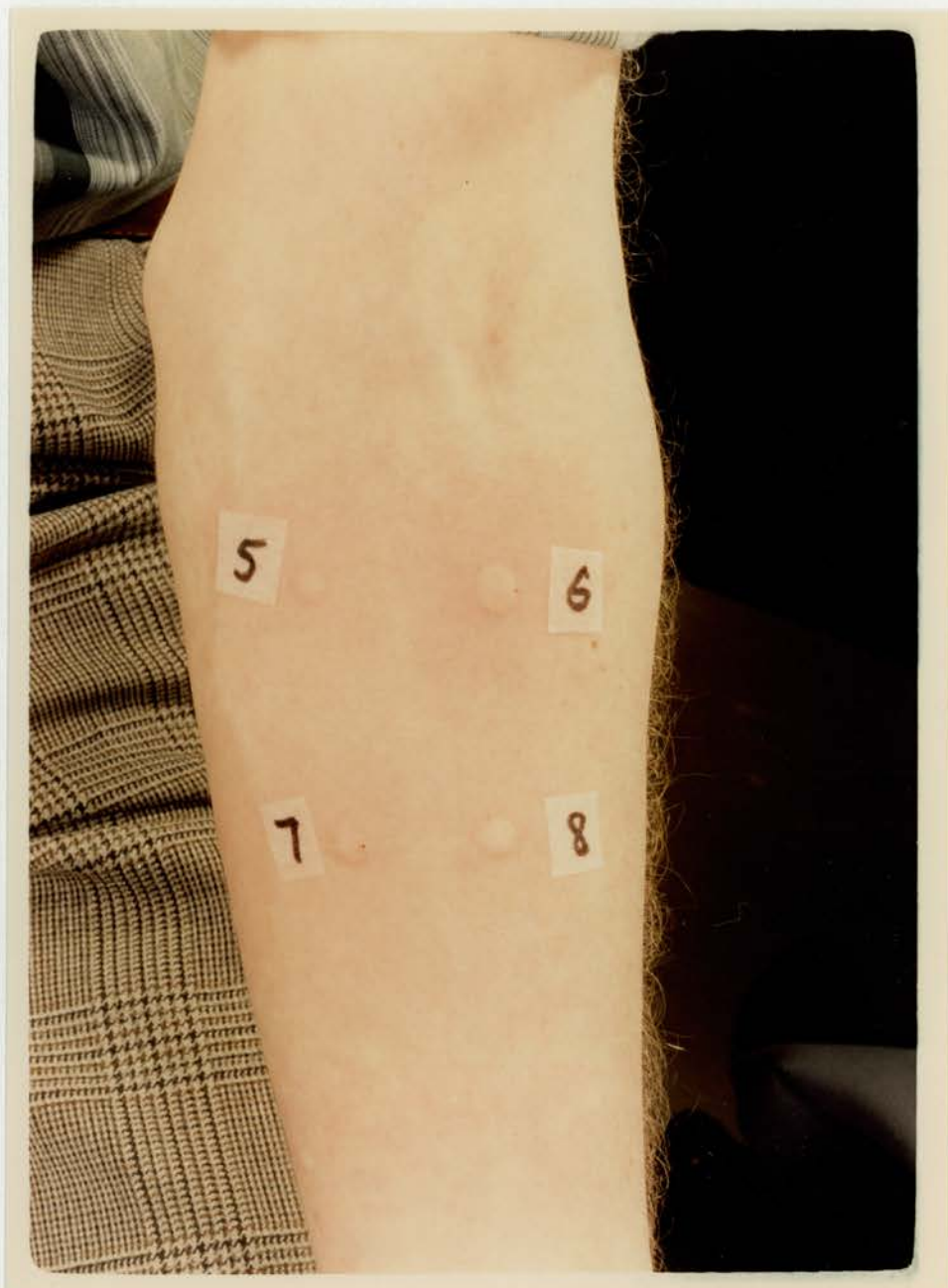


Fig 41. Wheals of different sizes after injection of coded solutions. Site 6 received ZAS and has a flare.

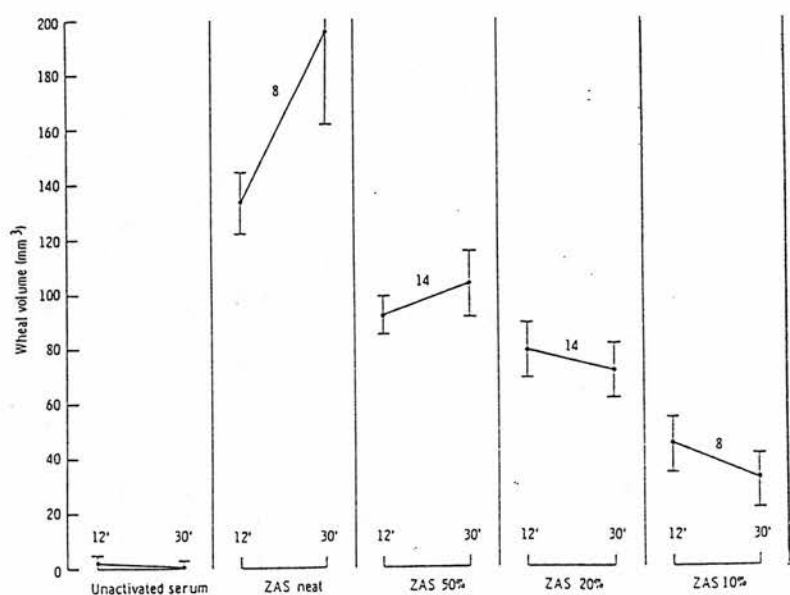


Fig 42. Time course of wheal response to ZAS
(mean ± SEM) (numbers are shown for each group).

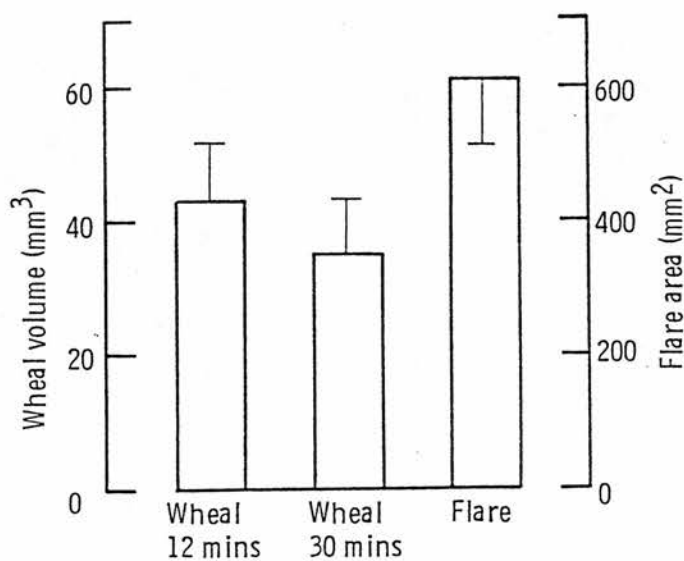


Fig 43. Wheal and flare response in normal subjects to the low molecular weight fraction (50%) of ZAS.
(mean ± SEM, n = 7).

wheal and flare response, albeit an attenuated one, suggests that the inflammatory mediators in ZAS are of low molecular weight.

Experiment 3 Wheal and flare responses to heated or C_5 depleted sera.

Method

A. Inactivation of complement

Sera were heated at 56°C for 30 minutes prior to or following zymosan activation.

B. C_5 depletion

Sera from normal subjects were depleted of C_5 using an affinity chromatography gel, as described in Chapter 2. Sera passed over the gel were negative for the hepatitis B surface antigen. Sera prepared by this method were shown by two-dimensional immuno-electrophoresis and Ouchterlony immunodiffusion to be devoid of C_5 while retaining C_3 . They were then zymosan activated as before. Statistical comparisons were made between normal and either heated or C_5 depleted sera by paired t test, but because the responses were not necessarily normally distributed, the non-parametric Wilcoxon's signed rank test was also used.

Results Removing C_5 from sera prior to zymosan activation resulted in a highly significant reduction in wheal volume at both 12 and 30 minutes (Fig 44). The p values refer to those obtained by paired t test; by Wilcoxon's signed rank test all pairs were significantly different at the 1% level. No flares were produced by C_5 depleted sera at any concentration.

Heating sera to 56° for 30 minutes prior to zymosan activation similarly prevented generation of wheal producing

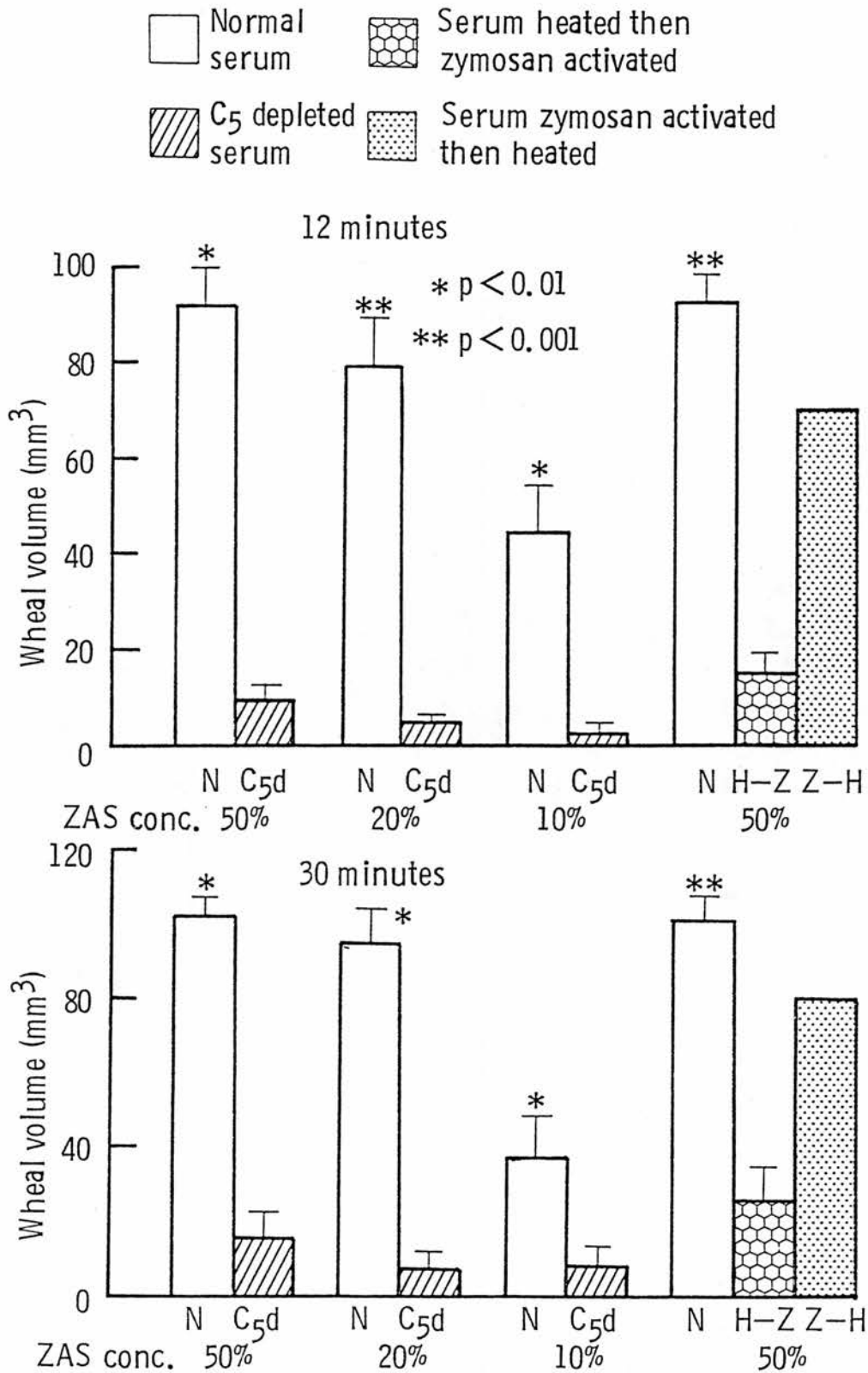


Fig 44. The effect of C₅ depletion or heating on generation of wheal-producing activity in the skin (mean \pm SEM, n = 8 except for zymosan activated then heated, where n = 2).

activity (Fig 44), and likewise such sera produced no flares. When sera were first zymosan activated then heated there was an approximately 25% reduction in wheal size, but this was not significant.

Experiment 4 Wheal and flare responses to ZAS, LMW fr and histamine in subjects with severe neutropenia.

(Studied with kind permission of Drs J Fletcher and Dr PAE Jones).

Methods Ten neutropenic subjects of both sexes with peripheral blood neutrophil counts of less than $0.5 \times 10^9/l$ were studied with their informed consent and hospital ethical committee approval. Their ages ranged from 16-72 (mean age = 50) and their diagnoses included marrow dysplasia or hypoplasia, hypersplenism, and neutropenia following chemotherapy for Hodgkin's disease or acute leukaemia (Table 11). All subjects were afebrile and free from infection when studied and no subject was taking antibiotics or anti-inflammatory agents. Subjects were injected with autologous ZAS, and its low molecular weight fraction (LMW fr) as described above.

To ensure that sera in the normal and neutropenic groups contained comparable amounts of complement components, C_3 and C_4 were measured turbidometrically, and total haemolytic complement was measured by a standard haemolytic assay in sera from normal and neutropenic subjects prior to zymosan activation (kindly arranged by Dr R J Powell). C_5 conversion products were measured in sera from neutropenic subjects following zymosan activation by the release of cobalamin-binding protein from normal cytochalasin B treated

neutrophils, as described in Chapter 2. This release appears to depend on C_5 conversion products. To ensure that the neutropenic patients were capable of producing reactive wheals and flares, 3 neutropenic subjects also received 50 μ l 1.2×10^{-4} M histamine intradermally.

Results In neutropenic subjects, the flare response to both concentrations of ZAS was significantly reduced (Fig 45), and the low MW fr produced no flares at all in the neutropenic group. The p values were obtained by both unpaired t test and Wilcoxon's signed rank test, which gave identical values. Wheal responses to ZAS were also significantly less in neutropenic subjects, both at 12 minutes (Fig 45) and at 30 minutes (Fig 46). The low MW fr also generated much less of a wheal response in the neutropenic group at both time points (Figs 45 and 46), although the difference at 30 minutes did not reach statistical significance. These findings suggest that PMN's are involved in producing both vasodilatation and an increase in vascular permeability.

There was no significant difference between normal and neutropenic subjects in the response to saline or unactivated serum (Fig 47), and the wheal and flare response to histamine in three neutropenic subjects was not impaired (Fig 48). Because these subjects found the histamine injections both painful and itchy, and because of the increased risk of skin infection, it was not felt justified to test the rest of the neutropenic group with histamine. Serum C_3 , C_4 and total haemolytic complement (CH_{50}) were normal in neutropenic subjects (Table 12) except for one (subject 7) who had slightly low C_3 and C_4 . His CH_{50} was normal and his skin

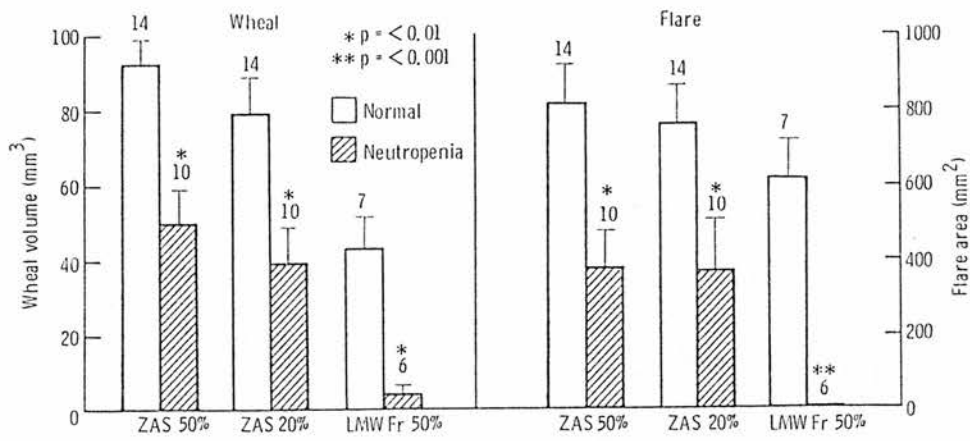


Fig 45. Wheal and flare responses at 12 minutes to ZAS and LMW fr. in normal and neutropenic subjects. (mean \pm SEM, numbers of subjects indicated above each bar).

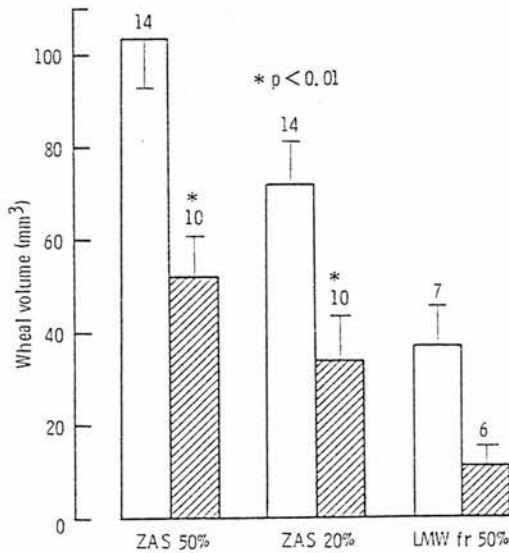


Fig 46. Wheal responses at 30 minutes to ZAS and low molecular weight fraction in normal and neutropenic subjects (numbers of subjects indicated above each bar). Mean \pm SEM shown in each case.

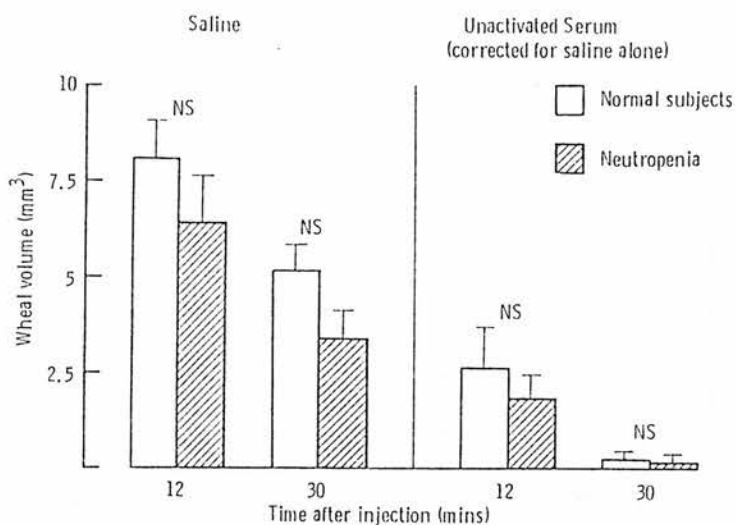


Fig 47. Wheal volumes 12 and 30 minutes after 50μl saline or 50% unactivated serum in normal and neutropenic subjects. (mean \pm SEM, 10 in each group).

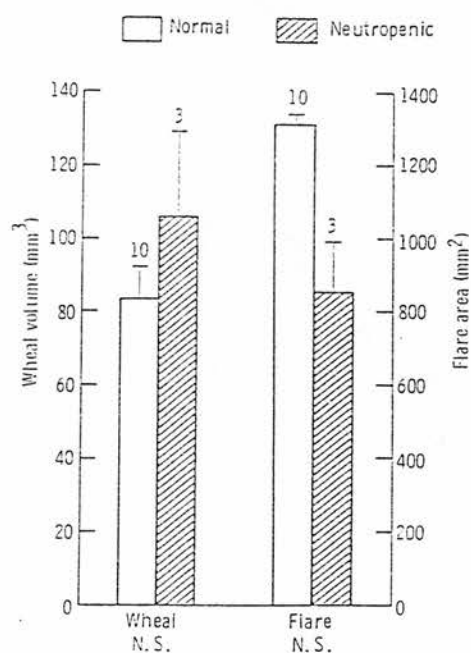


Fig 48. Wheal and flare responses 12 minutes after intradermal injection of 10⁻⁴M histamine in normal and neutropenic subjects. Results show mean \pm SEM in each case, with n indicated above each bar.

Table 12

Complement levels in unactivated serum from neutropenic subjects
(nos 1-10 are as in Table 1).

| Normal range | C ₃ 0.79-1.60g/l | C ₄ 0.12-0.36g/l | CH50 >180 |
|--------------|--------------------------------|--------------------------------|--------------|
| Subject 1 | 1.36 | 0.33 | Spec. broken |
| 2 | 1.16 | 0.11 | 370 |
| 3 | 0.87 | 0.21 | >350 |
| 4 | 1.37 | 0.25 | >350 |
| 5 | 1.35 | 0.20 | >350 |
| 6 | 0.77 | 0.08 | >350 |
| 7 | 1.22 | 0.13 | 331 |
| 8 | 1.21 | 0.32 | >350 |
| 9 | 1.48 | 0.32 | >350 |
| 10 | 1.35 | 0.24 | >350 |

responses were not lower than other neutropenic subjects. C_5 conversion products as assessed by degranulation of normal PMN's were similar in ZAS from normal and neutropenic subjects (Fig 49), so that the reduced inflammatory responses in neutropenic subjects could not be attributed to injection of reduced levels of complement derived mediators.

There appeared to be no influence of platelet count on the response, for when wheals and flares in those neutropenic subjects who were also thrombocytopenic (platelet count $< 50 \times 10^9/l$) were compared with those with platelet counts of $> 100 \times 10^9/l$ no significant differences were seen (Fig 50).

Experiment 5 Effect of prostaglandin E_2 (PGE_2) and ZAS together.

Method To see whether ZAS and PGE_2 together produced synergy of response, $50 \mu l$ injections were made of ZAS (10 and 20%) and prostaglandin E_2 ($50 \mu l$ of a 5.68×10^{-6} M solution, giving 3×10^{-10} mols/site, as previously described¹²¹). $50 \mu l$ of mixtures of ZAS and PGE_2 , containing the same final concentrations of each as when prepared separately, were also injected. Synergy was considered to be present if the response to ZAS + PGE_2 when injected together was significantly greater than the sum of the responses to the two substances injected separately.

Results When injected alone, PGE_2 produced small wheals, little bigger than saline alone, but with a noticable flare response disproportionate to the size of the wheal (bars A, Fig 51). There was no evidence of synergy with ZAS (Fig 51), for wheals and flares produced by the ZAS/ PGE_2 mixture (bars

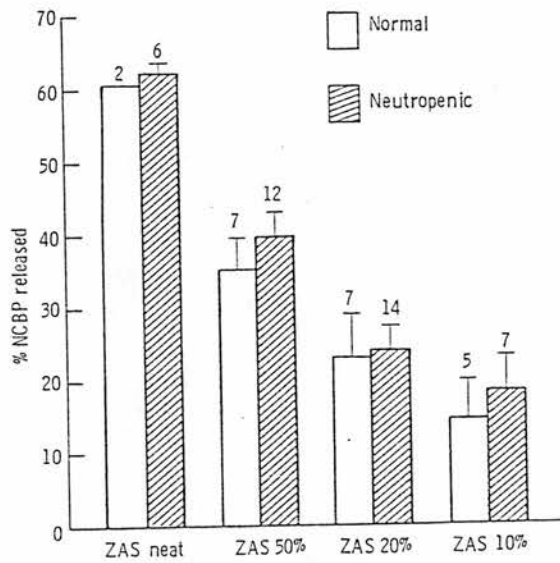


Fig 49. NCBP release from normal cytochalasin B treated PMN's by ZAS from normal and neutropenic subjects. (mean \pm SEM, n indicated in each case).

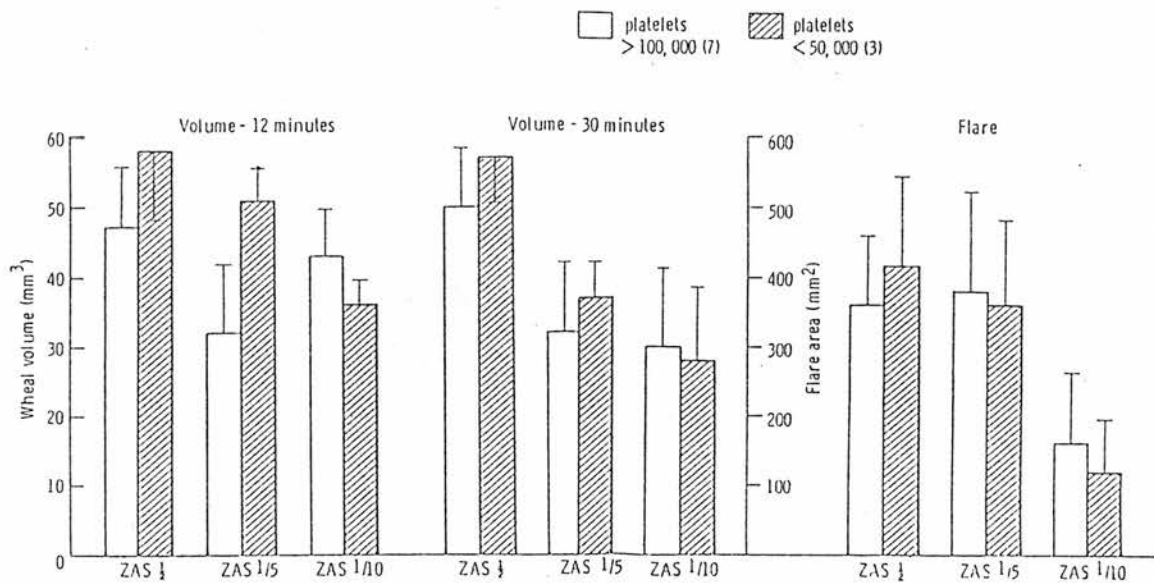


Fig 50. Effect of platelet count on response to ZAS in neutropenic subjects (n indicated in brackets above, mean \pm SEM shown in each case).

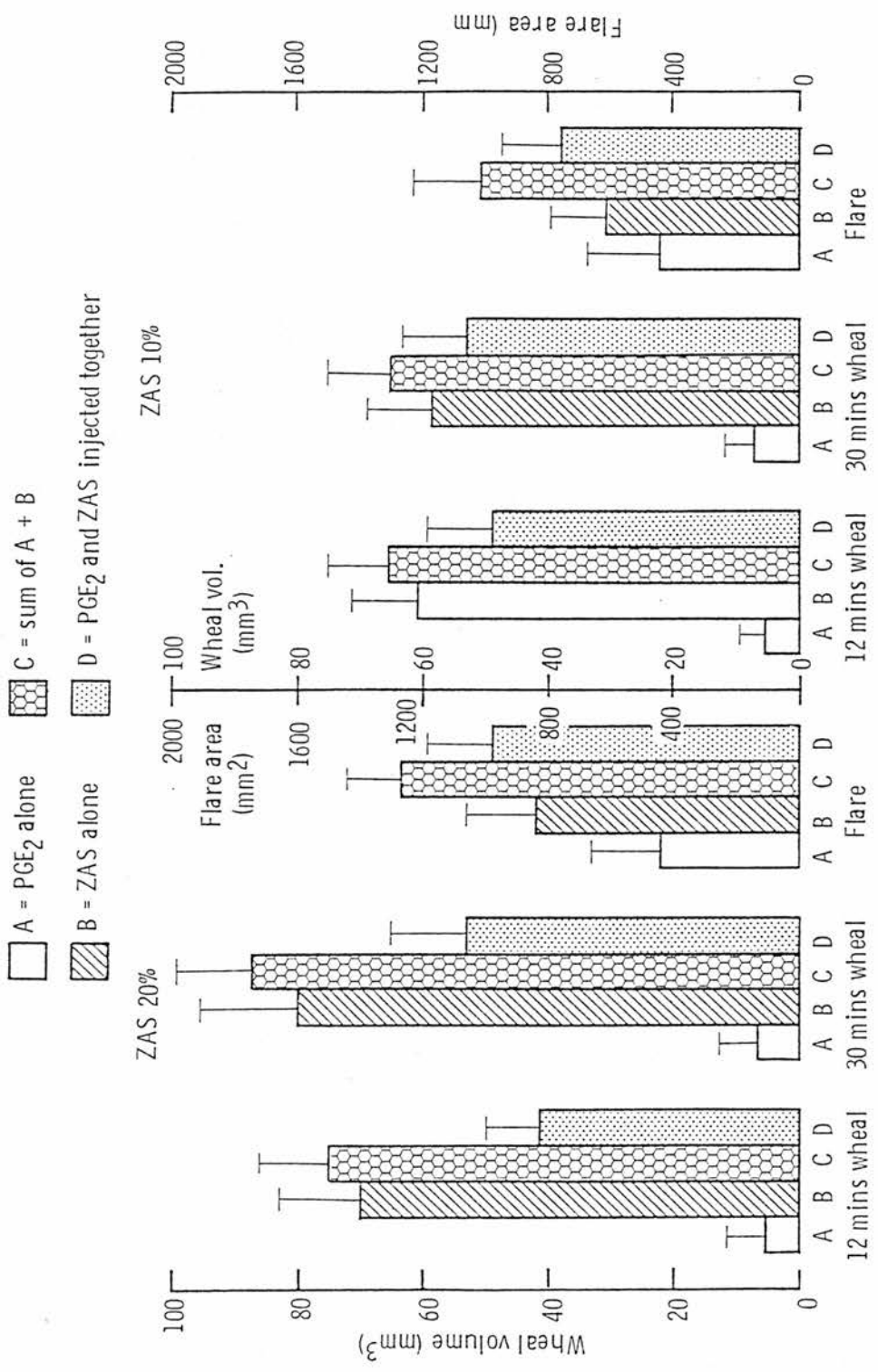


Fig 51. Wheal and flare responses to ZAS and PGE₂, injected together and separately (mean \pm SEM, n = 4).

D) were not greater than the sum of the responses to ZAS and PGE_2 injected separately (bars C). Indeed, responses produced by the mixture were always less than the sum of the separate responses, particularly wheals produced by the higher ZAS concentration, but this difference never reached statistical significance.

Experiment 6 **Biopsy of a wheal.**

Method Punch biopsy of a wheal was made 10 minutes after injection of 50 μl 50% ZAS in 1 subject (kindly performed by Dr Paul Maurice). The biopsy was taken into gluteraldehyde (to prevent mast cell degranulation), processed for light microscopy, and stained with haematoxylin and eosin.

Result There was obvious oedema in the tissues. The most striking feature was in the blood vessels as in Fig 52, which shows a number of PMN's adherent to the wall of what is probably a venule. There were no PMN's seen extravascularly.

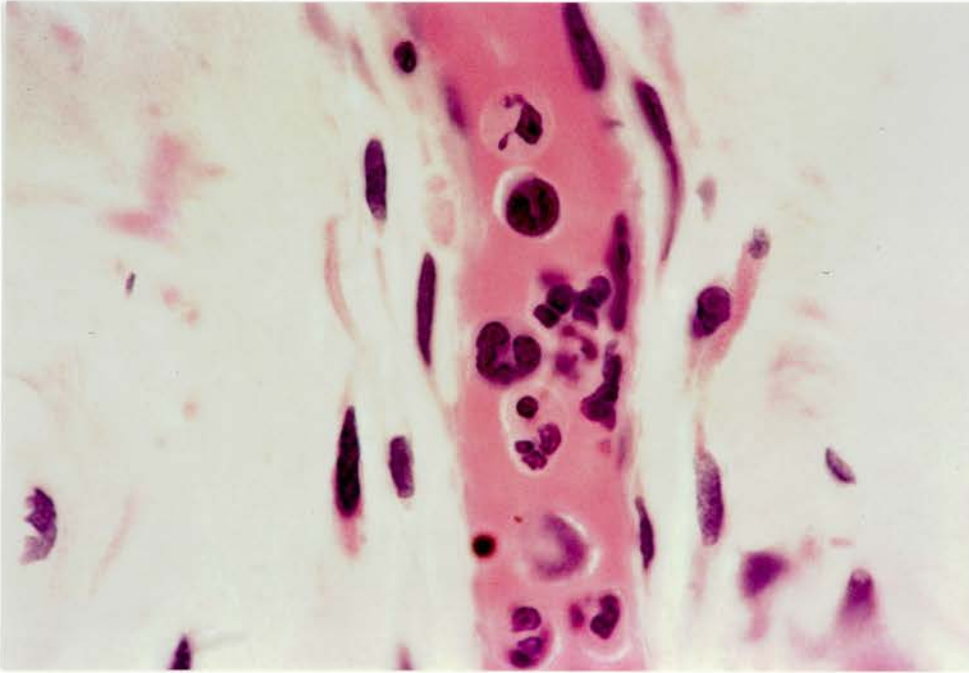


Fig 52. Light micrograph of biopsy of a skin wheal 10 minutes after injection of 50 μ l 50% ZAS, stained with haematoxylin and eosin.

This shows oedematous tissue with a venule containing an accumulation of PMN's apparently adherent to the vessel wall.

DISCUSSION

These studies have demonstrated the feasibility of using a simple skin model to assess the inflammatory activity of mediators. The technique of measuring wheal thickness using skin calipers has been evaluated by others^{154, 155}. Their data, and that presented here, shows that it is an acceptably reproducible method, provided that not too many measurements are made of each wheal during the response, for each act of measurement may compress its size by up to 10%¹⁵⁵. There is, however, considerable variation between subjects in their skin response to autologous ZAS. This is, surprisingly, no greater than that seen with a standard stimulus such as histamine (see Chapter 5), and therefore suggests that variations in basal complement levels and generation of vasoactive complement fragments are not as important as would be expected in producing the variability of the final response, which therefore appears to be much more dependent on factors inherent in the skin and blood vessels.

The observation that activated complement can cause local wheal and flare reactions in the skin is long established. This study has attempted to define the mechanism with respect to both the complement component(s) and cell type(s) involved. The results of the experiments with heated ZAS confirm a complement source for the inflammatory activity, with loss of wheal producing activity by heating sera before complement activation, as unactivated C_3 and C_5 are heat labile. There is much less loss of activity when sera are heated following complement activation, as complement split products are much more heat stable⁶⁰. Separation of ZAS into

high and low molecular weight fractions suggests that a major source of inflammatory activity lies in the molecular weight range of less than 30,000 Daltons. This low MW fraction, at the same concentration as the ZAS from which it was formed, produced much less inflammatory activity than whole ZAS, but as discussed in Chapter 2, not all small molecules can pass through the filter due to 'clogging up' by large molecules. For this reason, finding inflammatory activity in the 'high MW' fraction would actually have yielded little information about the molecular size of the inflammatory mediators.

C_5 depleted sera, despite the persistence of C_3 , produced very little wheal and flare reaction in the skin. C_3 , on maximal complement activation in the absence of EACA, would yield C_{3a} des Arg at a concentration of approximately $0.45-1.0 \times 10^{-5} M$, about 10 times the maximal concentration of C_{5a} des Arg⁶⁰. C_{3a} des Arg therefore appears to have no role in wheal and flare production in human skin, and the findings point to low molecular weight C_5 products being the main inflammatory mediator. The main such product generated when serum is activated without EACA is C_{5a} des Arg, formed from C_{5a} . It did not prove possible to test EACA-ZAS (which would contain mainly C_{5a}) in the skin, as the EACA itself was highly inflammatory.

What cell type(s) could C_{5a} des Arg be activating? The main contenders, from *in vitro* studies, are mast cells and PMN's. Although unconverted C_{5a} is a highly potent mast cell activator, the des Arg form, at least at concentrations achievable in serum, has little activity towards mast cells⁶⁰. It is possible that mast cells could be activated by any

unconverted C_{3a} or C_{5a} in ZAS, but in the absence of EACA removal of the arginine proceeds extremely rapidly. An alternative mast cell stimulus could be one or more of the PMN-derived mast cell triggering systems which have been described^{128, 156}. The possible role of mast cell histamine in the response is examined further in Chapter 5, but the notable lack of stinging and itching produced by intradermal ZAS is in marked contrast to histamine, even when the subjects do not know which injection is which, and suggests that any histamine component in the response to ZAS is relatively minor. Moreover, to invoke the mast cell as a major contributor to the reaction, it would have to be postulated that mast cells were in some way impaired in neutropenic subjects. Mast cells have a slow rate of turnover¹⁵⁷, and are therefore unlikely to be affected by alkylating agents or other chemotherapeutic drugs. Mast cell function in neutropenic subjects has not been formally tested, by, for example, intradermal injection of the mast cell degranulator 48/80, but there was no difference in response to ZAS between those who had had chemotherapy and those who had not.

The data can therefore best be explained by the hypothesis that the final wheal and flare response in some way involves circulating PMN's. Although reactions to ZAS were not totally abolished in neutropenic subjects, as they were in the mustine-treated neutropenic rabbits¹²¹, the peripheral blood counts in our subjects were often much greater than the 1% of normal obtainable experimentally¹²², for even a count of $0.5 \times 10^9/l$ is no less than 10% of normal. Concentrations of C_{5a} des Arg achievable in ZAS are well within the range which

activates PMN's, which is 10^{-8} - 10^{-7} M⁶⁰. The lack of wheal response to C₅ depleted serum in the skin is strikingly similar to the failure of C₅ depleted serum to attract PMN's into skin chambers in human subjects¹⁵⁸ and to its inability to degranulate PMN's *in vitro*. Thus wheal production and PMN activation seem closely linked. Indeed the size of the permeability response produced by chemotactic factors seems to correlate closely with the rate of PMN accumulation¹¹⁸.

The response to activated complement in man also differs from that in the rabbit in that ZAS is entirely effective in producing wheals and flares without the simultaneous presence of a vasodilator such as PGE₂. PGE₂ can be synergistic with permeability agents such as platelet activating factor in man¹⁵⁹, suggesting that basal blood flow in human skin is not rate-limiting to be permeability reaction. However, not only was there no synergy between ZAS and PGE₂, but the response to ZAS was actually reduced when PGE₂ was present, for reasons as yet unclear. It has been suggested that exogenous vasodilators are not required in man for the permeability response to ZAS to be seen, because a minor degree of mast cell activation might be occurring with release of histamine and leukotrienes A, C and D. This itself could dilate the vascular bed sufficiently to allow wheal production without the need for exogenous vasodilators (Williams TJ, personal communication).

The exact mechanisms of PMN involvement in the production of the vascular response to ZAS remain unclear, in terms of both how C₅ fragments and PMN's come into contact with each other, and how PMN's then contribute to the

permeability increase. Contact between C_5 fragments and PMN's could be explained either by diffusion of C_{5a} between endothelial cell junctions¹⁶⁰, or by transport of C_{5a} through the endothelial cell from the abluminal to the luminal side¹⁶¹. A further alternative mechanism is that the binding of C_5 fragments to the abluminal side of the endothelial cell leads to alterations in the characteristics of its luminal membrane, such that PMN's adhere to it.

There are many possible ways in which PMN activation might produce microvascular changes. Activated PMN's undergo degranulation and a respiratory burst, the possible role of which will be examined in Chapter 5. They also convert membrane lipids into products which have been implicated in the inflammatory response. Prostaglandin E_2 , as was observed in this study, certainly causes a small wheal and notable flare in the skin, confirming the observations of others¹²⁴. Thromboxane A_2 , on the other hand, causes vasoconstriction¹⁶², and the lipoxygenase product leukotriene B_4 (LTB₄), although a moderately powerful chemoattractant^{163, 164} and promotor of neutrophil adhesion to endothelium^{164, 165}, does not cause immediate wheals and flares in human skin^{166, 167} but a delayed, indurated reaction with neutrophil infiltration^{166, 168}. In animals, its effects in skin are neutrophil dependent^{121, 169}, and blockage of its synthesis does not impair the skin response to C_{5a} des Arg in the rabbit¹⁷⁰, so that although LTB₄ may have a role in amplification of the inflammatory response by recruitment of further PMN's, its synthesis by them is unlikely to play a major part in immediate wheal and flare production in response to C_5 .

fragments.

PAF-acether, on the other hand, which is produced by PMN's within minutes of exposure to C_{5a}^{171} , or C_{5a} des Arg¹⁷² does cause an immediate wheal and flare response in rabbit skin which is independent of circulating neutrophils¹²⁶, and has similar effects in man¹⁷³, where it is 100-1000 times more potent than histamine. Some subjects also show a late reaction to PAF-acether, with erythema and hyperalgesia, which we have not observed with ZAS, but it remains possible that PAF-acether is one of the permeability-producing mediators involved in the skin reaction to ZAS. An alternative source of PAF-acether is vascular endothelium¹⁷¹, although whether PMN adhesion could stimulate its synthesis remains to be seen.

PMN's could also cause capillary leakage by direct effects on the endothelial cell barrier. It has been proposed that the actual movement of PMN's through capillary or venule walls might open up tight junctions between the cells, allowing the escape of plasma¹²¹. This may not actually require the physical presence of the PMN's between the endothelial cells, but could result from impairment of endothelial cell integrity by PMN products. Oxidation products, for example, can cause K^+ efflux and purine release from endothelium¹²⁷. Adhesion to, or movement through, a tissue barrier do seem to be important in activating PMN release mechanisms. PMN's moving into sterile suction skin blisters released secondary, but not primary granule contents into the blister fluid¹⁷⁴, a phenomenon which may be important in renewing surface receptors. The permeability response to C_{5a} des Arg correlates closely in time with the period of PMN

adherence to and migration across the vessel wall¹¹⁸, while the presence of PMN's actually in the tissues seems to contribute little to the vascular response to these mediators. In this study, biopsy of a well-developed wheal showed many intravascular PMN's adherent to vessel walls, but none extravascularly, supporting the suggestion that PMN's activated during adhesion but prior to migration into the tissues can produce an increase in permeability. PMN's in the tissues might actually be refractory to further activation by the same stimulus, for stimulus-specific desensitisation of PMN's moving into inflammatory skin sites has been described¹⁷⁵.

C_{5a} des Arg might attract PMN's by sticking, not only to the PMN's but also to the vascular endothelium. The evidence is however conflicting, with pre-treatment of the endothelium with C_{5a} being reported to either increase¹⁷⁶ or have no effect on¹⁶¹ PMN adhesion. Once adherent to endothelium, PMN's could secrete factors such as oxidation products and PAF-acether, thus promoting both vasodilatation and microvascular permeability.

CHAPTER 5

STUDIES INTO THE MECHANISMS OF PMN INVOLVEMENT IN THE SKIN RESPONSE TO ACTIVATED COMPLEMENT

INTRODUCTION

As indicated in Chapter 4, there are many possible mechanisms by which PMN's could cause an increase in vascular permeability. The work in this chapter attempts to examine the role of some of these mechanisms.

The oxidase enzyme of PMN's, situated in the cell membrane, converts free oxygen to reactive tissue-damaging oxygen radicals, such as the superoxide anion, the hydroxyl radical and hydrogen peroxide. PMN's in certain inflammatory sites, such as those entering the lung in ARDS, show increased activity of this enzyme as demonstrated by chemiluminescence⁸⁴. PMN oxidative products may also be responsible for some of the damage to cartilage and synovium in rheumatoid arthritis¹⁰¹. *In vitro* studies using cultured endothelial cells^{127, 177} and perfused rabbit lungs⁸⁹ have suggested that these oxidative products might be important in increasing endothelial permeability to proteins.

The capacity of activated normal PMN's to increase albumin transfer across cultured endothelial cells appears to be absent in PMN's from individuals suffering from chronic granulomatous disease (CGD)¹⁷⁸, in which oxidase function is defective. To see whether the oxidase is equally important in

increasing endothelial permeability *in vivo*, we have examined wheal and flare responses to ZAS in an individual with CGD and in four female carriers of the condition.

A further situation in which functional and biochemical abnormalities of PMN's have been repeatedly described is chronic granulocytic leukaemia (CGL). These include impaired adhesion to glass¹⁷⁹ and nylon¹⁸⁰, reduced emigration to extravascular sites¹⁸¹, and defective phagocytosis¹⁸² and killing¹⁸³. Biochemically, low levels of lysozyme¹⁸⁴ and lactoferrin¹⁸⁵ have been observed, as well as the well characterised deficiency of alkaline phosphatase. In addition, there may be defects in metabolism of oxygen products, for reduced levels of hydrogen peroxide are formed during phagocytosis¹⁸⁶, and in response to soluble stimuli (Rule S, unpublished observations), though superoxide generation appears to be normal. CGL PMN's appear to degranulate normally during phagocytosis and in fact a greater percentage of granule enzymes can be recovered from the medium than when normal cells are used⁴⁴. Since normal PMN's degrade up to 20% of their primary granule enzymes by oxidation¹⁴⁰, the increased enzyme recovery from CGL cells may reflect reduced production of oxygen radicals⁴⁴. To see if these *in vitro* defects are mirrored in an *in vivo* response, the skin reactions to ZAS have been examined in CGL patients in the chronic phase of the disease.

The role of mast cells in the skin response to complement is investigated in two ways. Firstly a comparison of the reaction is made in the presence and absence of local and systemic anti-histamines of anti-H₁ type. Secondly, the

possibility that activated PMN's can trigger mast cell release of histamine *in vitro* is examined, as previously suggested by the observations of Stendahl¹²⁸ and Coble¹⁵⁶.

Finally the possibility that stable inflammatory products, such as prostaglandins and leukotrienes, contribute to the reaction is tested by examining the skin reaction following systemic corticosteroid, aspirin, or non-steroidal anti-inflammatory (NSAI) drug administration. Steroids act by preventing the release of arachidonic acid from membrane phospholipids, thus blocking both cyclooxygenase and lipoxygenase pathways, while NSAI's in standard doses inhibit the cyclooxygenase pathway. The supernatant from actively phagocytosing PMN's is also examined for wheal producing activity in the skin to see whether stable inflammatory mediators can be detected and identified.

EXPERIMENTAL WORK

Experiment 1 Skin responses to ZAS and histamine in subjects with chronic granulomatous disease (CGD).

(Studied with kind permission of Drs WG Reeves, JA Raeburn and OB Eden)

Methods One male case of CGD (aged 9) and four female carriers of the condition (including the mother of the case) received intradermal injections of 50 μ l of autologous ZAS 50%, 20% and 10%, LMW fr from autologous ZAS, and histamine 1.2×10^{-4} M. Wheal and flare responses were measured at 12 and 30 minutes as before. All subjects had been previously investigated and found to have either impaired reduction of nitroblue tetrazolium, reduced chemiluminescence, or both. Individuals were well when studied, and on no antibiotic therapy.

Results No definite conclusions could be drawn. The case of CGD was able to produce wheals and flares in response to both ZAS and histamine. Wheals in response to both stimuli were well below normal, (Fig 53), but there were no age matched controls. Flare responses, on the other hand, were above normal to both stimuli (Fig 53). The carriers also showed normal flare responses to both stimuli, and normal wheal production to histamine (Fig 53). Wheal responses to ZAS appeared to be reduced, but whether this is a real finding is unclear on such a small sample.

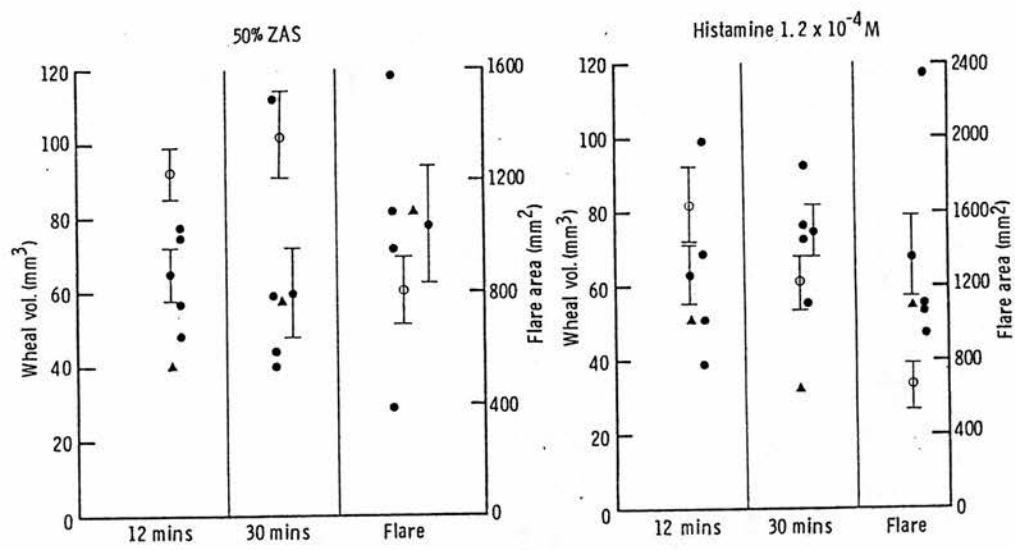


Fig 53. Wheal and flare responses to 50% ZAS and histamine in a case of CGD (▲) and 4 female carriers (●).
Mean (± SEM) of carriers = Mean (± SEM) of 10 normal subjects =

Experiment 2 Skin responses to ZAS and histamine in

subjects with chronic granulocytic leukaemia (CGL).

(Studied with kind permission of Drs J Fletcher, PAE Jones, DR Ryrie, T E Bletcher and E A French)

Methods Nine subjects of both sexes, aged 34-68, previously diagnosed as having CGL, were tested as above. All were in the chronic phase of the disease - for details see Table 13. All subjects received 50 μ l of dilutions of autologous ZAS and LMW fr, as well as histamine 1.2×10^{-4} M as control. Serum C_3 , C_4 and CH_{50} was measured in unactivated serum from each subject as described in Chapter 4, and C_5 conversion products were assayed in ZAS by release of NCBP from normal PMN's as described in Chapter 2.

Results There was no difference between normal and CGL subjects in wheal size after saline or unactivated serum (Fig 54). Similarly, the wheal size 12 minutes after histamine was no different in the two groups (Fig 54), although resolution was slower in the CGL group, which also showed a slightly greater flare response to histamine. Neither of these differences, however, reached statistical significance.

Wheal and flare responses to 50% and 20% autologous ZAS were, however, significantly reduced in subjects with CGL (Fig 55). Responses to 10% ZAS were also reduced, but only the flare response was significantly less (Fig 56). The biggest difference was in the reaction to 50% LMW fr (Fig 56), with minimal wheal formation and no flare response at all in CGL subjects. These differences could not be accounted for by reduced levels of baseline complement components in CGL group, for all subjects had normal C_3 , C_4 and CH_{50} levels (Table 14),

Table 13

Clinical details of patients with chronic granulocytic leukaemia (CGL)

| Age | Sex | Hb (g/dl) | WCC ($\times 10^9/l$) | PMN's ($\times 10^9/l$) | Platelets ($\times 10^9/l$) | Ph ¹ Pos | Treatment |
|-----|-----|-----------|----------------------------|------------------------------|----------------------------------|------------------------|--------------------------------|
| 1 | F | 12.1 | 34.0 | 20.5 | | yes | Busulphan |
| 2 | F | 13.2 | 36.2 | 24.4 | 171 | yes | Busulphan |
| 3 | F | 13.1 | 20.1 | 15.5 | 743 | yes | Busulphan |
| 4 | M | 15.1 | 14.7 | 8.5 | 394 | yes | Busulphan Prev. Splenectomy |
| 5 | M | 17.6 | 6.9 | 2.9 | 178 | yes | Busulphan 6 Thioguanine |
| 6 | M | 13.8 | 31.9 | 21.1 | 193 | yes | Busulphan |
| 7 | M | 15.3 | 29.5 | 21.2 | 140 | yes | Busulphan |
| 8 | M | 14.6 | 14.9 | 12.1 | 906 | yes | Busulphan |
| 9 | F | 13.1 | 24.6 | 14.0 | 689 | yes | Nil |

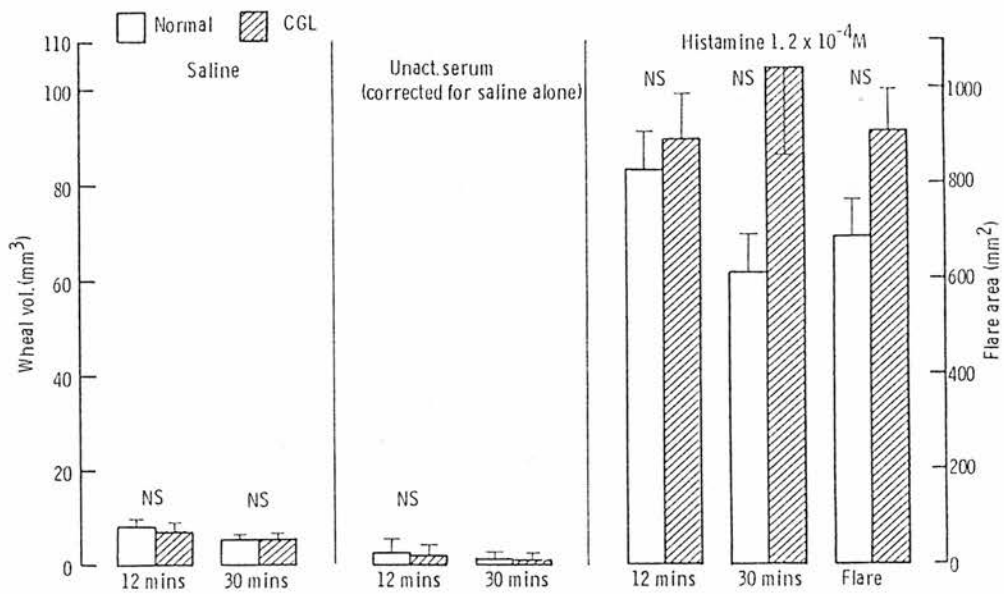


Fig 54. Wheal responses to saline and unactivated serum (50%) and wheal and flare responses to histamine, in normal subjects ($n = 10$) and subjects with CGL ($n = 9$), (mean \pm SEM) NS - No significant difference between groups.

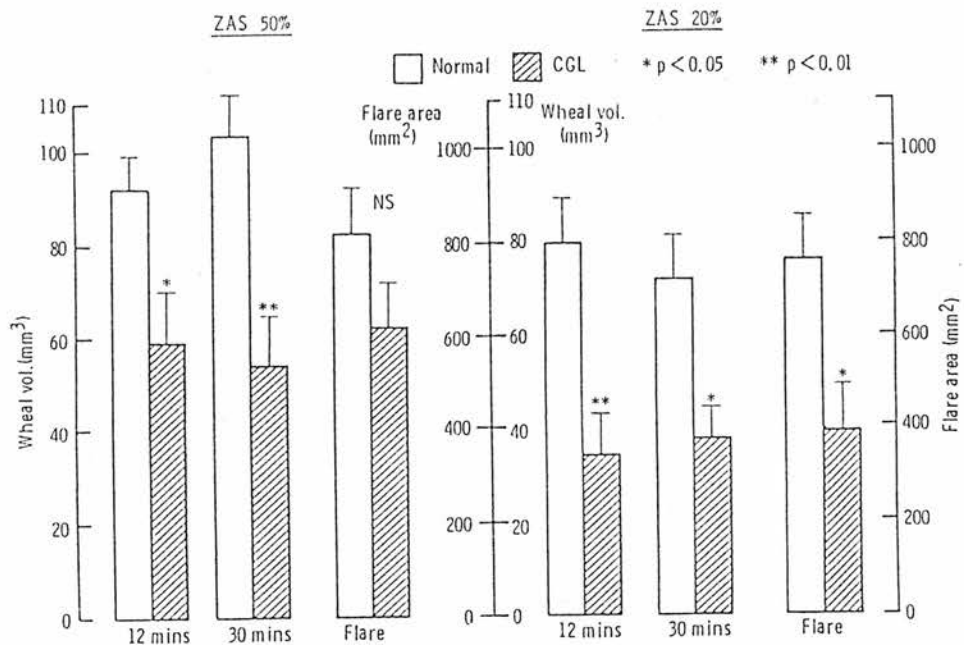


Fig 55. Wheal and flare responses to 20% and 50% autologous ZAS in normal subjects ($n = 14$) and subjects with CGL. (mean \pm SEM, $n = 9$) NS = No significant difference.

Table 14

Serum complement levels in patients with CGL

| | C ₃ | C ₄ | CH50 |
|--------------|----------------|----------------|------|
| Normal range | 0.79-1.60g/l | 0.12-0.36g/l | >180 |
| Subject 1 | ND | ND | >350 |
| 2 | 1.11 | 0.26 | >350 |
| 3 | 1.43 | 0.28 | >350 |
| 4 | 1.41 | 0.27 | 295 |
| 5 | 1.20 | 0.27 | 285 |
| 6 | 1.29 | 0.35 | >350 |
| 7 | 1.09 | 0.17 | >350 |
| 8 | 1.20 | 0.22 | >350 |
| 9 | ND | ND | 309 |

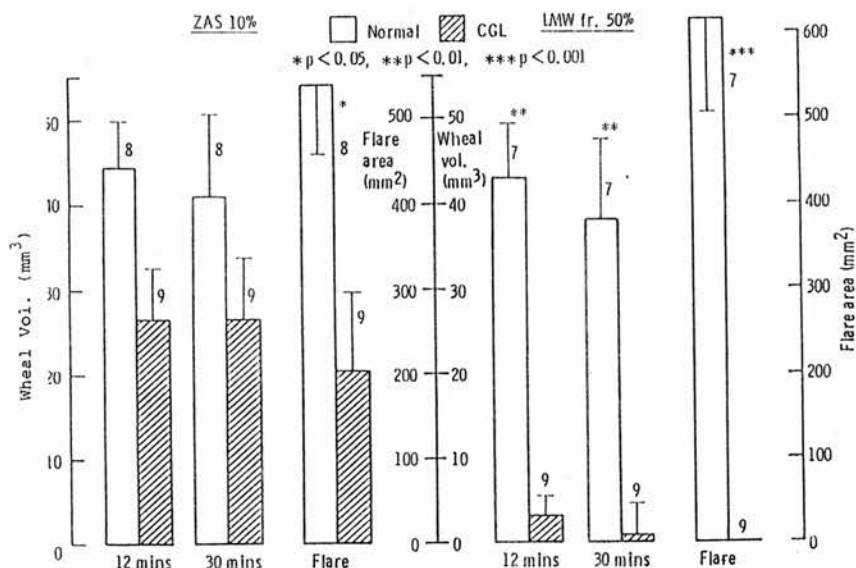


Fig 56. Wheal and flare responses to autologous ZAS (10%) or low molecular weight fraction (LMW fr. 50%) in normal subjects or subjects with CGL. (Number of subjects is beside each bar). Mean \pm SEM is shown in each case.

and C_5 conversion products in dilutions of ZAS were no different to those from normal subjects (Fig 57).

Experiment 3 The effect of local and systemic anti-histamine drugs (H_1 antagonists) on the wheal and flare response to intradermal ZAS.

Methods

(A) Local antihistamines

Ten normal subjects were injected at three separate sites with 50 μ l of either saline, clemastine $2 \times 10^{-4}M$, or clemastine $2 \times 10^{-5}M$ (all solutions coded). Thirty minutes later, as previously described¹⁸⁷, each site was reinjected with histamine (50 μ l of $1.2 \times 10^{-4}M$) and the wheal and flare response measured as before. Having ascertained that these concentrations of clemastine produced inhibition of histamine-induced reactions, 10 subjects were injected were injected with saline or clemastine as before, followed at 30 minutes by 50 μ l 50% autologous ZAS at the same sites. Reactions were measured as above.

(B) Systemic antihistamines

The wheal and flare reactions to 50 μ l of dilutions of ZAS and to 50 μ l histamine $1.2 \times 10^{-4}M$ were measured in six subjects before and after clemastine 1mg bd orally for three days. The second set of tests was done two hours after the last tablet.

Results

(A) Local antihistamines

When injected into the skin 30 minutes before histamine the H_1 antagonist clemastine caused significant reductions of

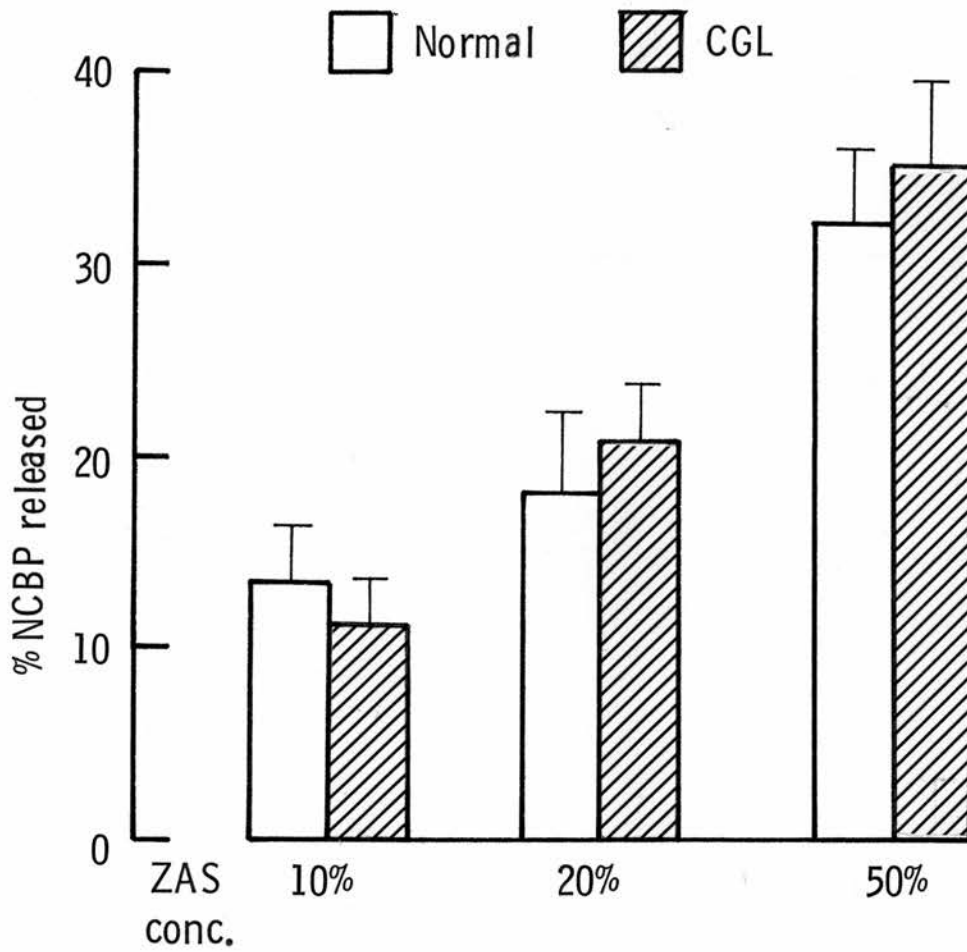


Fig 57. NCBP release from normal cytochalasin B treated PMN's by ZAS (without EACA) from normal subjects (n = 10) or subjects with CGL (n = 9)
Mean \pm SEM shown for each group.

both wheal volume and flare area when compared to sites injected with saline prior to histamine (Fig 58). Both concentrations of clemastine were effective, but inhibition was much greater at the higher concentration. When ZAS was the stimulus, clemastine again caused a significant reduction in both wheal and flare (Fig 59), but this time both drug concentrations were equally potent with regard to their wheal-inhibiting properties, with only a slight dose effect seen in flare inhibition. Interestingly, however, clemastine caused much greater inhibition of wheal production when the stimulus was histamine than when it was ZAS, particularly at the higher drug concentration (Fig 60). Histamine wheals were reduced in volume by about 80%, but ZAS wheals by only about 40%. This difference did not hold true for flare responses (Fig 60), with histamine and ZAS induced flares being inhibited identically by clemastine at either concentration.

(B) Systemic antihistamines

As with local administration of the drug, oral clemastine caused significant inhibition of the flare reaction to two concentrations of histamine, and to dilutions of ZAS (Fig 61), although this did not always reach statistical significance. There was little difference between the stimuli in the degree of inhibition achieved by the drug. Reduction of wheal responses by oral clemastine was much less apparent for both stimuli than when the drug was injected locally (Fig 62) and was highly significant only at the highest concentration of either stimulus. The reduction in responses also differed from local use of the drug in being similar in degree for the two stimuli.

□ Saline ▨ Clemastine 2×10^{-5} M ▤ Clemastine 2×10^{-4} M
 * $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$ when drug compared to saline

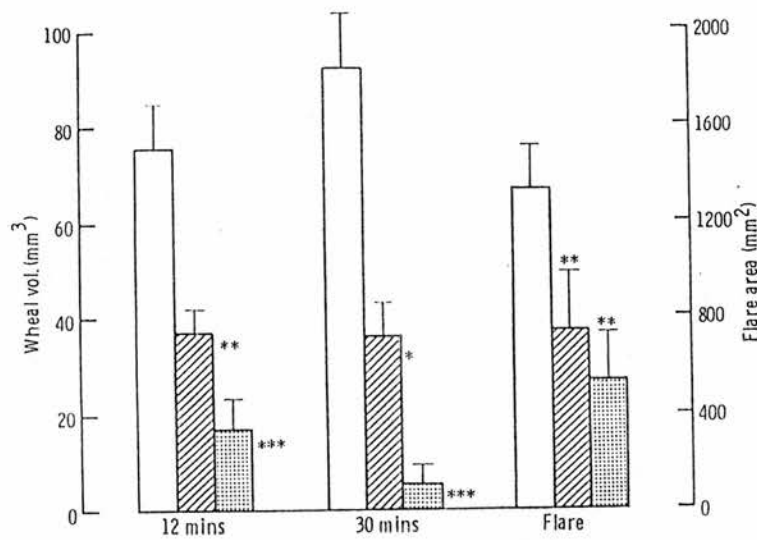


Fig 58. Wheal and flare responses produced in normal subjects by histamine 1.2×10^{-4} M in the presence of either saline or clemastine 2×10^{-4} M or 2×10^{-5} M. (mean \pm SEM, $n = 10$).

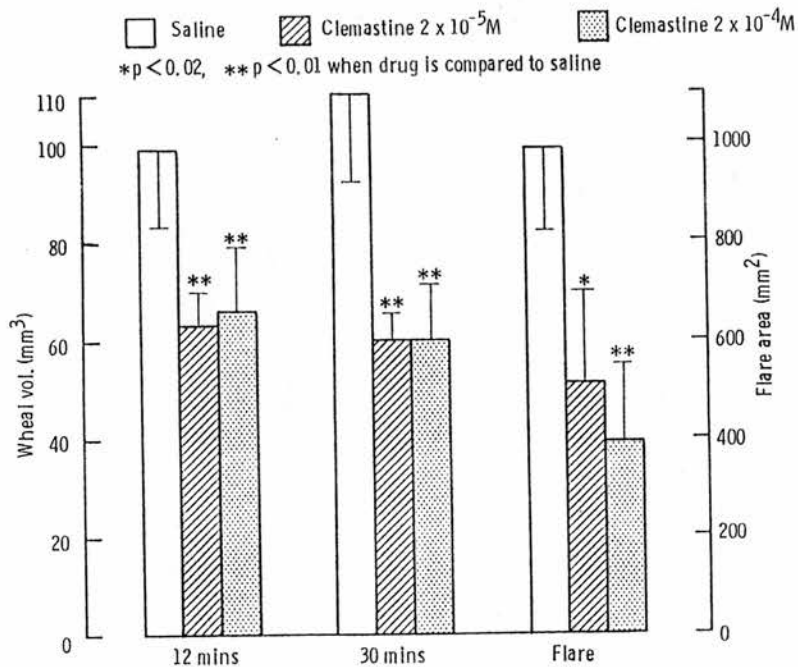


Fig 59. Wheal and flare responses produced in normal subjects (mean \pm SEM, $n = 10$) by 50% ZAS in the presence of either saline or clemastine 2×10^{-4} M or 2×10^{-5} M.

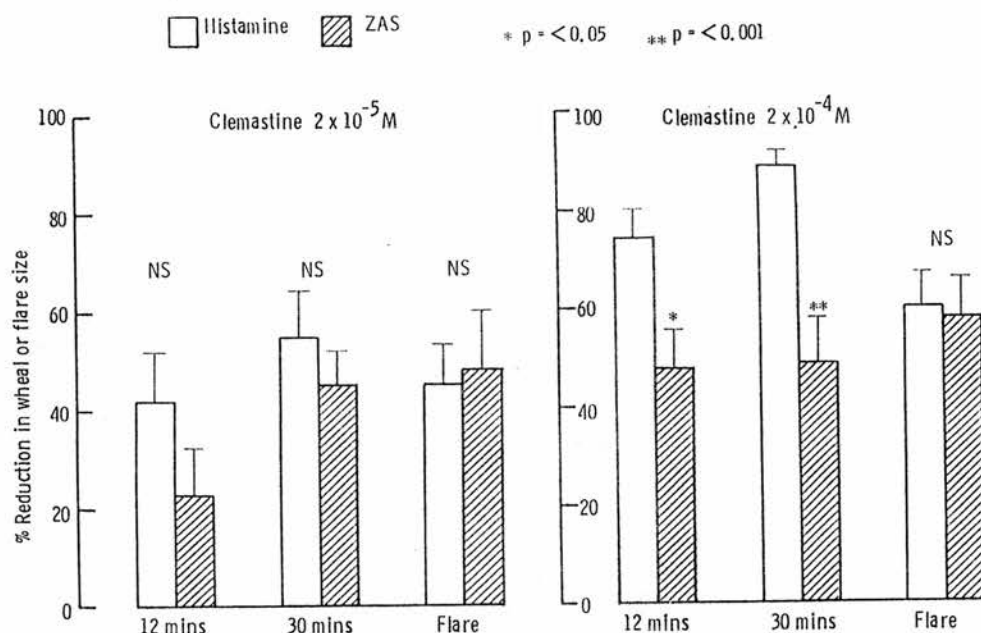


Fig 60. Percentage reductions in wheal and flare size by clemastine ($2 \times 10^{-5} M$ and $2 \times 10^{-4} M$) in response to either histamine ($1.2 \times 10^{-4} M$) or 50% ZAS.
(mean \pm SEM, n = 10) NS = No significant difference.

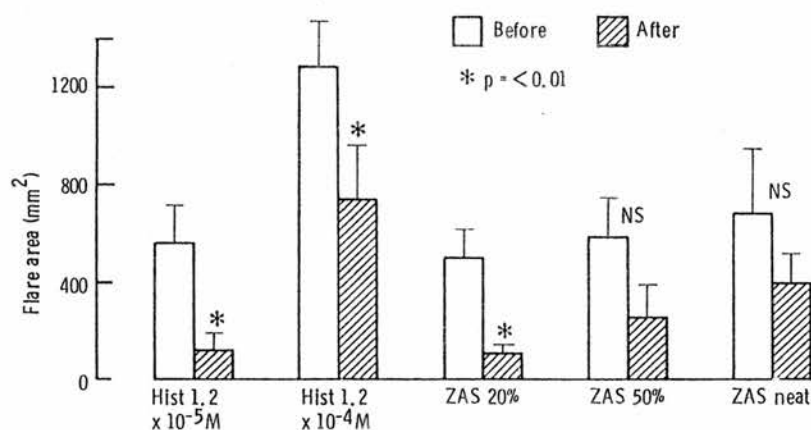


Fig 61. Flare areas produced by histamine and ZAS before and after 3 days of oral clemastine 1mg bd (mean \pm SEM, n = 6) NS = no significant difference between pairs.

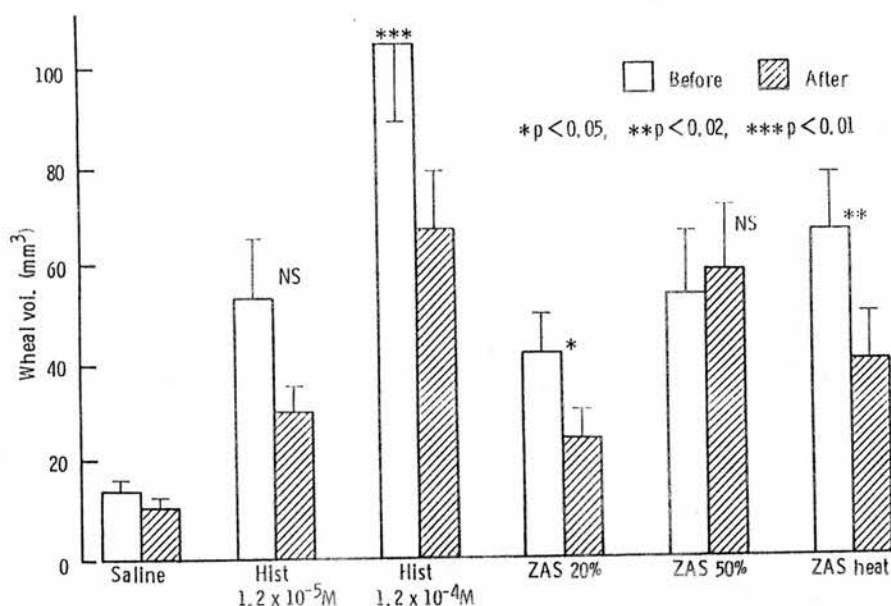


Fig 62. Wheal volumes produced by saline, histamine and ZAS before and after 3 days of oral clemastine 1mg bd (mean \pm SEM, n = 6). Measurements at 12 mins only.

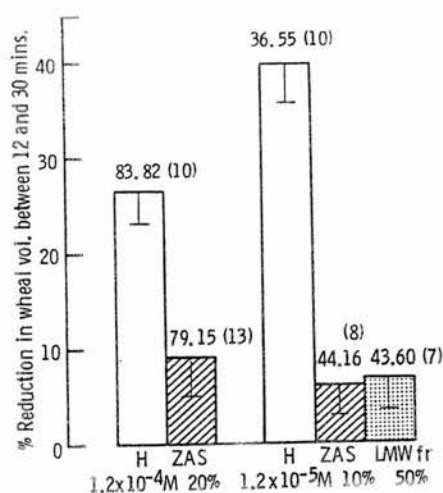


Fig 63. Percentage reduction in wheal volume between 12 and 30 minutes in wheals of comparable initial volume produced by different stimuli. Mean wheal volume at 12 minutes (mm³) is above each column followed by (n). Mean \pm SEM shown in each case.

While measuring wheals during these studies on antihistamines, a further difference between the responses to histamine and ZAS became apparent, namely the rate at which the wheals resolved. Histamine wheals reduced in volume by 25-40% between 12 and 30 minutes (Fig 63), while ZAS wheals of comparable size reduced by only 10-13% over the same time period.

Experiment 4 The effect of activated PMN's on histamine release from mast cells.

(kindly helped by Dr B Gomperts, Dept of Experimental Pathology, University College Hospital, London).

Methods Human PMN's were isolated using LSM and dextran, as described in Chapter 2. Mast cells were obtained from the peritoneal cavities of adult male Wistar rats by lavage with Hepes buffer (pH 7.2) containing Mg^{++} 1mM, Ca^{++} 1.7mM, D-glucose 5mM and 0.1% albumin. After washing and counting, mast cells (5×10^6 /tube) were suspended in buffer and the PMN degranulating stimuli added - FMLP, LMW fr or ZAS.

The PMN suspension was pre-exposed to cytochalasin B (final concentration $5\mu g/ml$) for 20 seconds and aliquots of the suspension then added to the tubes containing the stimulus and mast cells such that the final PMN concentration was 2×10^6 cells/tube. The stimulated PMN's and mast cells were incubated together for 5 minutes at room temperature. The supernatants were then removed for assay of histamine and in some cases, NCBP. Histamine was assayed by a standard fluorometric method¹⁸⁸. Control tubes contained mast cells, cytochalasin \pm stimuli, but no PMN's.

Results PMN's incubated with mast cells were not prevented from responding to the degranulating stimuli, as assessed by their release of NCBP (Table 15). This PMN activation did not result, however, in any increase in histamine secretion above that seen in unstimulated suspensions alone.

It is of interest that concentrations of both ZAS and LMW fr capable of causing wheal and flare reactions in the skin did not trigger release of any histamine from mast cells. At the end of the experiment, the mast cells were still viable, and secreted histamine normally in response to the stimulus 48/80.

Experiment 5 The effects of the anti-inflammatory drugs, aspirin, hydrocortisone and ibuprofen on the skin response to ZAS and histamine.

Methods Eighteen normal subjects were injected with 50 μ l intradermal histamine (1.2×10^{-4} M) and ZAS (20% and 50%), and wheal and flare responses measured as before. Six subjects in each group then received the following:-

- A. Hydrocortisone 100mg by intravenous bolus injection
- B. Aspirin 600mg q.i.d. for three days
- C. Ibuprofen 400mg t.i.d. for three days.

Group A then received an identical set of skin injections, beginning 30 minutes after the injection of hydrocortisone. Groups B and C received the repeat skin injections on day 4, two hours after the last tablet.

Results

A. Hydrocortisone

Hydrocortisone caused no change in haemoglobin or

Table 15

Release of histamine from mast cells incubated with
PMN's and a PMN degranulating stimulus, and
release of PMN NCBP (mean of 2)

| | % histamine release | | % NCBP release |
|--------------------|---------------------|--------------------|-------------------|
| | Mast cells alone | Mast cells + PMN's | |
| 1. FMLP | | | |
| none | 0.68 | 2.21 | |
| 10 ^{-9.5} | 1.36 | 1.22 | |
| 10 ⁻⁹ | 0.68 | 0.54 | |
| 10 ^{-8.5} | 1.63 | 5.58 | |
| 10 ⁻⁸ | 1.09 | 1.36 | |
| 10 ^{-7.5} | 0.95 | 1.77 | 37.3 |
| 10 ⁻⁷ | 1.77 | 1.36 | 37.2 |
| 10 ^{-6.5} | 0.95 | 1.77 | 39.1 |
| 2. ZAS (no EACA) | | | |
| none | 0.00 | 0.00 | |
| 10 ⁻³ | 0.00 | 0.27 | |
| 10 ^{-2.5} | 0.00 | 0.00 | |
| 10 ⁻² | 0.00 | 0.00 | |
| 10 ^{-1.5} | 0.00 | 1.22 | |
| 10 ⁻¹ | 0.81 | 1.77 | 49.58 |
| 10 ^{-0.5} | 0.47 | 0.45 | 53.77 |
| 10° (neat) | 0.00 | 0.00 | 40.31 |
| 3. Low Mw Fraction | | | |
| none | 0.00 | 0.00 | |
| 10 ⁻³ | 0.00 | 2.18 | |
| 10 ^{-2.5} | 0.68 | 0.00 | |
| 10 ⁻² | 0.00 | 0.00 | |
| 10 ^{-1.5} | 1.50 | 3.26 | |
| 10 ⁻¹ | 2.04 | 2.18 | 47.28 |
| 10 ^{-0.5} | 0.00 | 0.00 | 48.24 |
| 10° (neat) | 0.00 | 0.00 | 38.72 |

platelet concentrations, but produced the well documented neutrophilia and lymphopenia, (Fig 64). An unexpected finding was the highly significant monocytopenia, which has also been previously described¹⁸⁹.

Hydrocortisone intravenously did not however cause any changes in the wheal and flare reactions to 50% or 20% ZAS (Fig 65) or to histamine (Fig 66).

B. Aspirin

Aspirin produced no changes in wheal responses to 20% or 50% ZAS (Fig 67). The flare areas after both ZAS concentrations were reduced by about 25%, but this was not statistically significant. There was likewise no significant reduction in the wheal and flare response to histamine by aspirin (Fig 68).

C. Ibuprofen

Ibuprofen did not affect skin responses to either 50% or 20% ZAS (Fig 69) or to histamine (Fig 70).

Experiment 6 The skin response to intradermal injection of medium from phagocytosing PMN's.

Principle

When PMN's phagocytose a bacterium or inert particle, a proportion (50-90%) of the secondary granule contents appears extra-cellularly⁴⁴. The stimulus (if particulate) and the cells can then be removed, leaving a neutrophil conditioned medium (PNCM) containing granule contents and other PMN secretory products.

Method

A. By phagocytosis of *Candida guilliermondii*

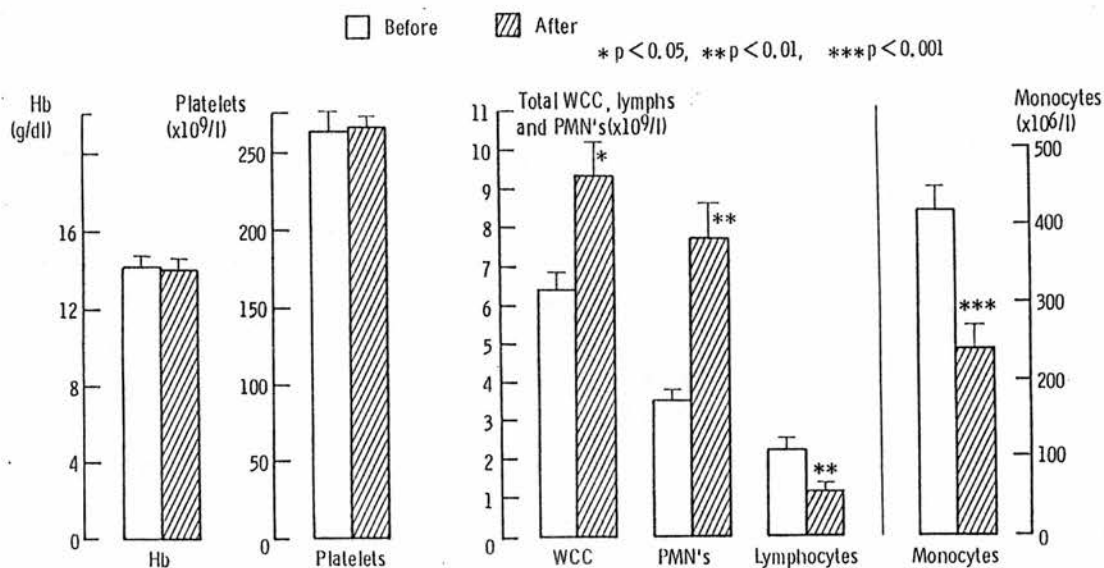


Fig 64. Changes in haematological parameters in normal subjects (mean \pm SEM, $n = 6$) before, and 30 mins after hydrocortisone 100mg intravenously.
Hb - haemoglobin; WCC - White Cell Count; PMN's - neutrophils

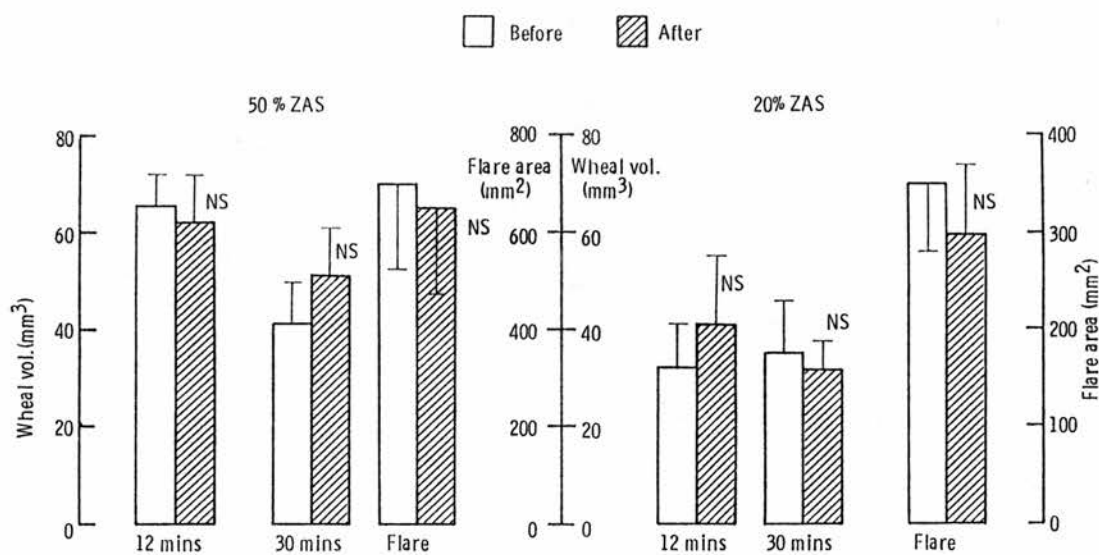


Fig 65. Wheal and flare responses in normal subjects to 20% and 50% ZAS, before, and 30 mins after, intravenous hydrocortisone 100mg. (mean \pm SEM, $n = 6$).

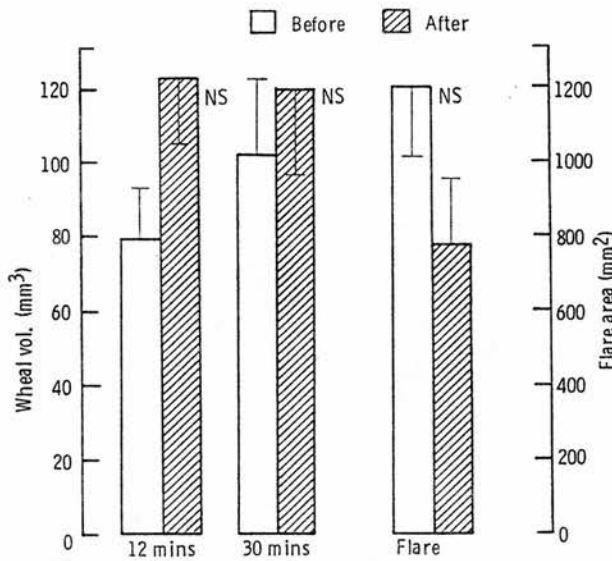


Fig 66. Wheal and flare response to histamine ($1.2 \times 10^{-4} M$) in normal subjects before, and 30 mins after intravenous hydrocortisone 100mg. (mean \pm SEM, n = 6)

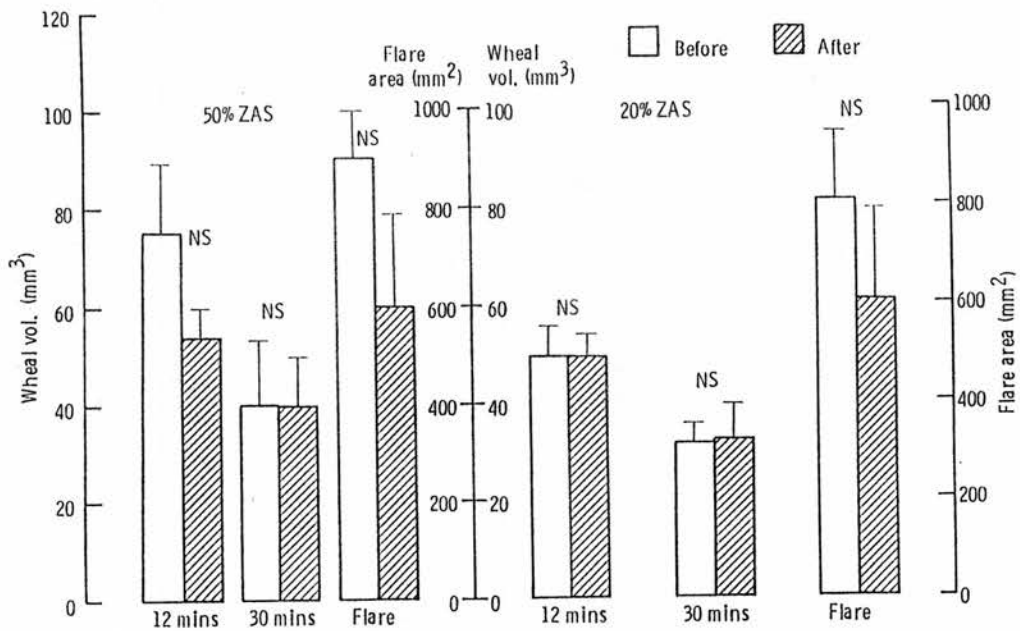


Fig 67. Wheal and flare responses produced by 20% and 50% ZAS in normal subjects before and after aspirin 600mg qid for 3 days. (mean \pm SEM, n = 6).

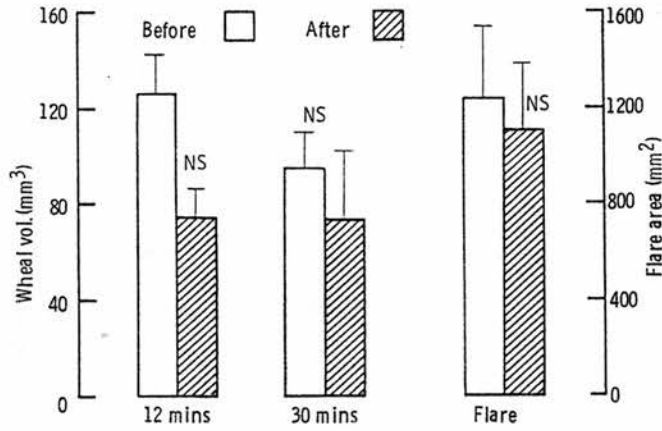


Fig 68. Wheal and flare response to histamine $1.2 \times 10^{-4}M$ in normal subjects before and after aspirin 600mg qid for 3 days. (mean \pm SEM, n = 6)

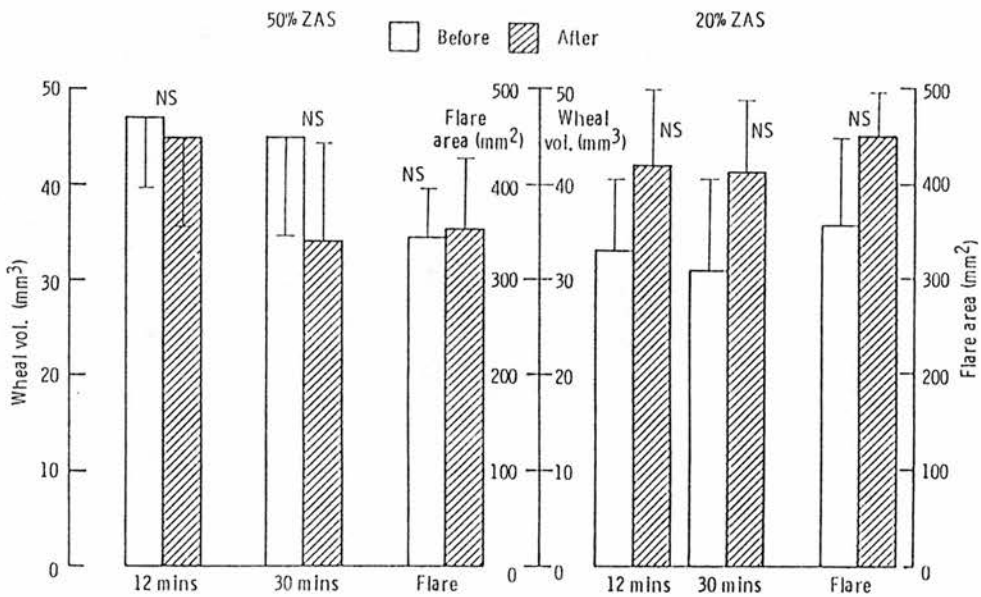


Fig 69. Wheal and flare responses to 20% and 50% ZAS in normal subjects before and after ibuprofen 400mg tid for 3 days. (mean \pm SEM, n = 6). NS = No significant difference between groups.

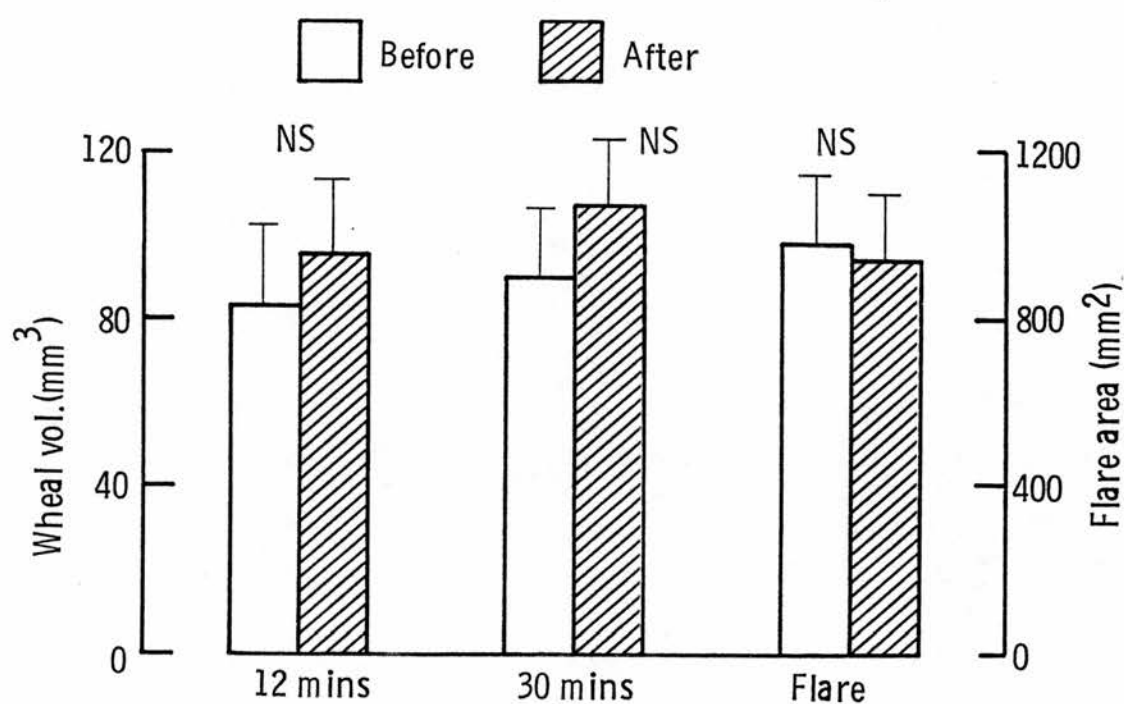


Fig 70. Wheal and flare response to histamine $1.2 \times 10^{-4}M$ in normal subjects before and after ibuprofen 400 mg tid for 3 days (mean \pm SEM, $n = 6$).

PMN's from the subject to be tested were incubated at a concentration of $12.5 \times 10^6/\text{ml}$ with $62 \times 10^6/\text{ml}$ *Candida guilliermondii* for 60 minutes at 37°C on a rotator. These conditions have already been shown to allow maximal phagocytosis of *Candida*¹⁸⁶.

The cells and *Candida* were removed by centrifugation at 1700g at room temperature for 10 minutes followed by passage through a 0.2μ filter. The supernatant was stored under liquid N_2 till injection.

B. By phagocytosis of latex beads

2mls of latex beads ($10^{10}/\text{ml}$, mean diameter 0.8μ) were opsonised by incubation with 2mls of the subject's serum at 37°C on a rotator for 60 minutes. The beads were washed twice in PBS by spinning for 10 minutes in a microcentrifuge and then resuspended in PBS containing 1mM Ca^{2+} and 0.7mM Mg^{2+} but no albumin. Because of losses during washing, the beads were recounted at this stage in a counting chamber and mixed with the subject's PMN's at a bead: cell ratio of 50:1 (cell conc = $5 \times 10^7/\text{ml}$; bead conc = $2.5 \times 10^9/\text{ml}$). The cell/bead mixture was incubated at 37°C for 60 minutes on a rotator and the cells and beads removed by spinning in a microcentrifuge followed by passage through a 0.2μ filter as before.

Control supernatants were prepared from tubes containing cells alone, and opsonised beads alone. To ensure that phagocytosis had occurred, a cytospin preparation was stained with Leishman stain. To assess PMN secretion during phagocytosis, an aliquot of PMN's was sonicated and NCBP release during phagocytosis was assessed by measurement of NCBP in the PNCM and in the sonicate.

Results Initial experiments used *C. guilliermondii* as the phagocytic stimulus. The PNCM thus generated had a very small degree of wheal-producing activity in the skin (Fig 71, Exp 1) but several days later the skin sites became red, itchy and scaley. This was presumed to be due to pre-existing anti-Candida antibodies in the subject reacting with unremoved Candidal antigens in the PNCM, which could possibly also have accounted for the small immediate inflammatory response. For this reason, subsequent preparations of PNCM were performed with latex beads as the phagocytic stimulus. These latex beads were actively taken up by the PMN's (Fig 72) associated with release of about 40% of the total NCBP (Table 16). Despite this, the PNCM so produced did not result in any wheals and flares when injected into the skin of two different subjects (Fig 71, Exps 2 and 3). No delayed reactions were seen.

Table 16

NCBP levels from PMN sonicates and in the
Supernatants from PMN's undergoing phagocytosis
of opsonised beads

| | Experiment 1 | Experiment 2 | |
|---------------|--------------|--------------|--------|
| Sonicate | 4896.3 | 2413.9 | (ng/l) |
| Cells & Beads | 1873.4 | 1073.5 | |
| % son | 38.26% | 44.47% | |

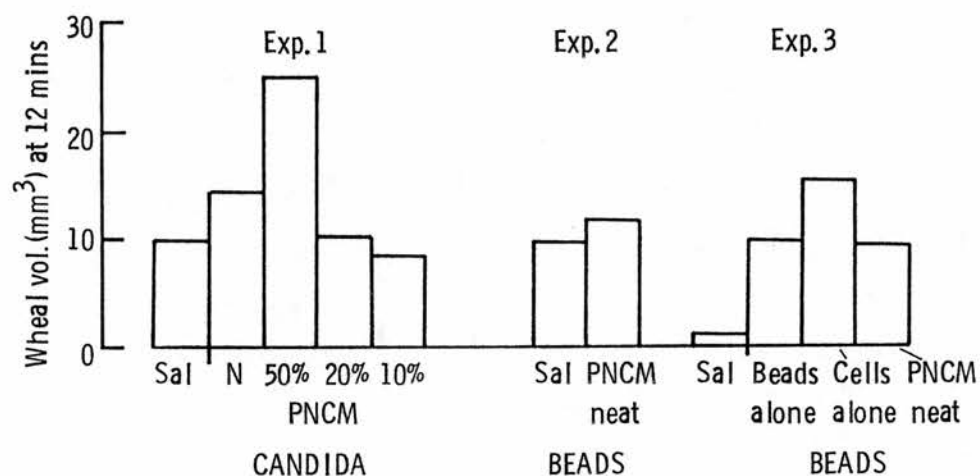


Fig 71. The effects of the supernatant from phagocytosing PMN's (PNCM) injected intradermally.

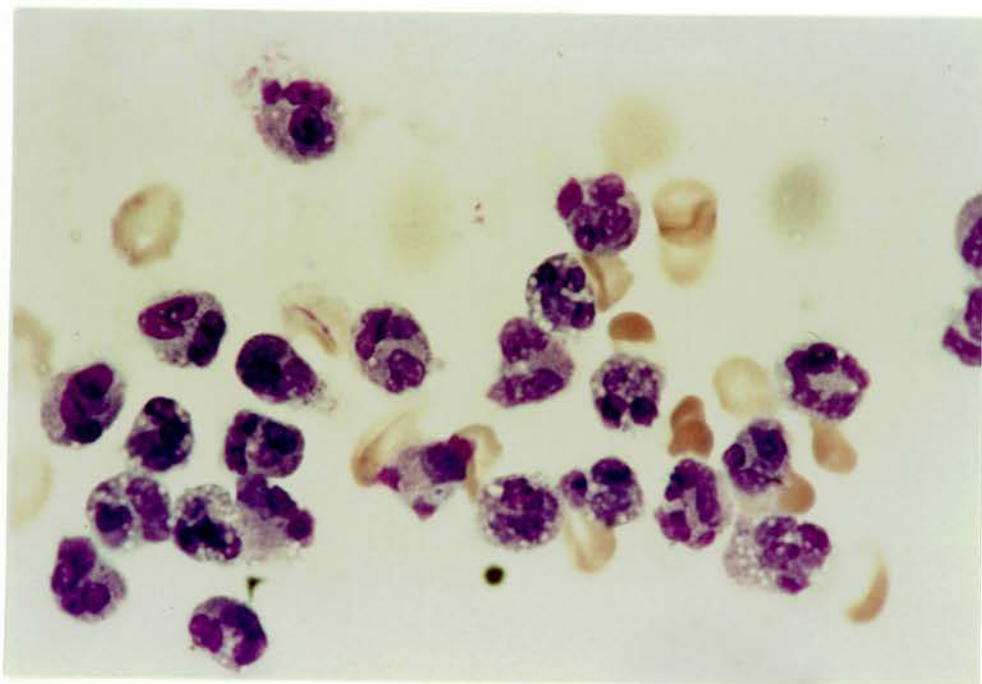


Fig 72. Cytospin preparation of neutrophils showing ingested latex beads in the cytoplasm of nearly every cell. Some cells contain 15-20 beads. (Leishman stain).

DISCUSSION

The studies in this chapter have attempted to delineate the mechanism(s) by which PMN's might act to produce a wheal and flare response in the skin, and although no definite conclusions have been reached, some interesting points have emerged.

The results with CGD subjects have proved difficult to interpret. The fact that the subject with CGD, known to have very low superoxide production, can generate a wheal and flare of any size in response to ZAS, suggests at least that oxidative metabolism of PMN's is not the sole mechanism involved. While oxidants may be the main source of permeability producing activity in some *in vitro* models^{127, 177}, clearly other mechanisms also operate *in vivo*. For example, CGD PMN's can synthesise thromboxanes¹⁹⁰ and leukotrienes¹⁹¹ normally *in vitro*, and while these may not themselves be the mediators involved, it does suggest that deficiency of the oxidase does not render the cell incapable of inflammatory activity. Had more CGD subjects been tested, clear differences between them and normals might have become apparent but this remains speculative.

The CGD carriers showed normal histamine reactions and a normal flare response to ZAS, but there is a suggestion that wheal production by ZAS was impaired. By the Lyon hypothesis, whereby one X chromosome in every cell in females is rendered inactive, these subjects should have 50% normal and 50% affected PMN's in the circulation. This means that the two cell populations may not behave identically with respect to adhesion to endothelium or migration to inflammatory sites.

However, the normal cells seem to be able to compensate in terms of bacterial defence, since these carriers gave no history of increased tendency to infections, and could therefore presumably compensate for the defective cells in the overall response to complement. Studies on greater numbers of subjects would be required to ascertain this.

Subjects with CGL showed a clearcut impairment of both wheal and flare response to ZAS, with normal responses to histamine, and normal complement levels. As with the neutropenic subjects, the defect was particularly obvious when LMW fr was the stimulus. These observations do not in fact help in elucidating the PMN mechanism(s) involved in generating a permeability change, for the defects described in CGL cannot be localised to one area of cell metabolism. In addition to the defects described above, impaired lipooxygenase activity¹⁹², and failure of release of inhibitors of myeloid colony growth¹⁹³ have been described. These new findings may, like the others, reflect a defect in stimulus-response coupling in CGL cells, which, even when the disease is controlled by drugs, are still found in the marrow and peripheral blood. Such a defect does not seem to lead to impaired host defence, but might give clues to the mechanism of the abnormal control of granulopoiesis seen in the condition.

The studies using local antihistamines were done to see whether mast cell activation could be implicated in the skin response to ZAS. Local clemastine was effective in greatly reducing histamine wheals and flares, as previously reported¹⁸⁷. The drug also had some inhibitory effects on ZAS

induced wheals and flares, unlike its effects in the rabbit, in which the response to C_{5a} was not affected at all by H_1 antagonists¹²¹. The degree of inhibition seen in the human subjects was similar for ZAS and histamine flares. However, ZAS wheals were reduced much less than those caused by histamine.

It could be argued, therefore, that flares are produced by either stimulus through the same mechanism, one which can be partly inhibited by clemastine and which therefore might involve release of mast cell histamine. This argument would not hold for wheals, since the inhibition produced by the drug was so different for the two stimuli. The results suggest rather that a histamine independent mechanism might be at least partly responsible for the permeability response to ZAS. This histamine-independent system might involve PMN's.

Interpretation of the results, however, is rendered even more complicated by the observations on the inhibitory effects of H_1 antagonists on PMN degranulation, as will be described in Chapter 6. An alternative explanation of the *in vivo* data is that the inhibitory effects of clemastine on skin responses to ZAS are due to its actions on PMN's, not its anti-histamine properties. In addition, the H_1 blocker brompheniramine injected locally also appears to inhibit wheals produced by the PAF analogue AcGEPC¹⁹⁴, so that antagonism of PAF secreted from activated PMN's might be a yet further action of H_1 antagonists in this system. The inhibition of PMN's by H_1 antagonists *in vitro* however, requires high drug concentrations, which although they can be injected locally into the skin cannot be achieved in plasma by oral

administration of the drug. Histamine antagonism, however, is seen with concentrations achievable orally. Thus the results of studies using oral clemastine, where both histamine and ZAS responses were partially reduced, does suggest that the inhibition seen with local clemastine is not solely due to PMN inactivation, but that histamine antagonism might also be taking place.

A further piece of evidence to suggest that histamine release is probably not the only permeability-producing effect of ZAS is that the kinetics of the responses to the two stimuli are different. Williams et al¹²¹ observed that C_{5a} wheals had a $t_{1/2}$ for resolution of 90-100 minutes, while that for histamine wheals was only 4-6 minutes. Such estimations were not possible using our method of measurement, for each single thickness measurement using calipers can itself reduce volume by up to 10%¹⁵⁵. However, by comparing wheal sizes for the two stimuli at 12 and 30 minutes, it was possible to confirm that ZAS wheals of comparable initial volume had decreased in size by 30 minutes much less than histamine wheals. This suggests that ZAS had induced a much more sustained mechanism of permeability production, supporting the idea that a histamine-independent mechanism is operating. The kinetics of response of whole ZAS appear similar to those of pure C_{5a} or C_{5a} des Arg¹⁹⁵.

A further hypothesis tested was that activated PMN's themselves triggered mast cell secretion. This was considered possible because at least two such mechanisms have been described, one from the myeloperoxide- H_2O_2 -halide system of PMN's¹²⁸ and the other requiring other oxidative metabolites,

but not MPO¹⁵⁶. PMN cationic proteins have also been implicated in mast cell activation¹⁹⁶. This hypothesis was particularly interesting, because it might have explained the impaired responses in CGL, where H_2O_2 production appears to be deficient¹⁸⁶. Under the conditions tested, however, no PMN activation of mast cells occurred, despite normal PMN secretion of NCBP and mast cell responsiveness to the degranulating stimulus 48/80. There are two possible explanations for this. One is that the incubation period of mast cells and PMN's together was too short and another is that the ratio of PMN's to mast cells was too low. A study which appeared after this experiment was performed suggested that a 40-60 minute incubation period was necessary¹⁵⁶, admittedly with non-cytochalasin treated PMN's, although detectable amounts of histamine were present by 10 minutes. The PMN : mast cell ratio of 0.5:1 (as used here) was probably far too low, as ratios of 10:1 - 50:1 seem to be optimum¹⁵⁶. An alternative approach would be to prepare PNCM, concentrate by lyophilisation if necessary, and test the concentrated solution for mast cell degranulating activity.

An important incidental observation in the experiment with mast cells was that neither ZAS nor LMW fr containing the des Arg forms of C_{3a} and C_{5a} , caused any PMN-independent release of mast cell histamine, in contrast to the potent mast cell releasing properties of unchanged C_{3a} and C_{5a} . As the mast cells were of rat origin, it cannot be absolutely certain that these preparations would have no activity for human mast cells, but the findings do support the hypothesis that mast cell involvement in the skin response to ZAS is probably only

minor.

The complete lack of inhibition of skin reactions by any of the three anti-inflammatory drugs tested is not totally unexpected. The concentrations of these drugs achievable in plasma by conventional doses are insufficient to produce the inhibition of PMN secondary granule release⁹¹ and superoxide production¹⁹⁷ seen with high concentrations of ibuprofen and indomethacin *in vitro*. There has been one report (published after this study was completed), suggesting that PMN's from subjects taking ibuprofen orally can show reduced FMLP-induced lysozyme release¹⁰¹, but the doses of drug used were twice those taken by the subjects reported here. Interestingly, even the higher dose of ibuprofen failed to inhibit FMLP-induced superoxide production. Any inhibitory effects produced by these drugs, therefore, would primarily reflect inhibition of cyclooxygenase, in the case of aspirin and ibuprofen. The actions of hydrocortisone will be discussed below. In the rabbit, the increase in blood flow and vascular permeability and leucocyte infiltration caused by intradermal *E coli*¹⁹⁸ or zymosan activated plasma¹⁹⁹ could be reversed by systemic indomethacin or local acetylsalicylic acid. However, administration of exogenous PGE_2 into the skin reversed not only the changes in blood flow and vascular permeability, but restored the leucocyte infiltrate also. Since PGE_2 is not of itself chemotactic, the inference is that the numbers of leucocytes present reflect solely the changes in flow and/or permeability, and are not themselves influenced by the drugs. As Williams has shown, lesions produced by intradermal C_{5a} in the rabbit depend on the simultaneous presence of vasodilator

prostaglandins¹²¹. As discussed in Chapter 4, this requirement for prostaglandins appears to be much less important in the response to C_{5a} in man, and is clearly not a rate limiting step. Thus prostaglandins appear to play only a minor role in the vascular response to activated complement in this system. The significance of the finding of increased PGI_2 production from endothelial cells exposed to hydrogen peroxide from activated PMN's is therefore unclear²⁰⁰.

The actions of hydrocortisone are more complex. The rapid production of neutrophilia is mainly due to movement of marginated PMN's from the endothelial surface into the circulating pool. There is also a small contribution from young PMN's which emerge from the marrow, although these do not appear to differ from peripheral blood PMN's in their granule contents³. Hydrocortisone also acts at a cellular level to inhibit the membrane phospholipase which makes arachidonic acid and other lipids available for synthesis of prostaglandins, leukotrienes and platelet activating factor. It is not clear whether these cellular effects would be in operation only 30 minutes after a bolus injection of hydrocortisone, but, as seen from the peripheral blood counts, the effects on PMN distribution had occurred. It is generally accepted that PMN activation and secretion takes place only when the cells are on a suitable surface²³, such as endothelium. Despite its effects on marginated PMN's hydrocortisone does not seem to have prevented this, and of course the stimulus of extravascular ZAS may well have overridden any hydrocortisone-induced tendency of the cells not to adhere to the endothelium. Similar results have been

obtained in the rabbit where much larger doses of hydrocortisone failed to inhibit the increased blood flow and vascular permeability or the leucocyte infiltration stimulated by ZAS²⁰¹.

Anti-inflammatory drugs seem therefore to have very little role in inhibiting the actions of activated complement in this system, consistent with their limited usefulness in states of generalised complement activation such as ARDS.

The final experiments in this chapter attempted to find permeability-producing activity in supernatants from phagocytosing PMN's. Phagocytosis is associated with a respiratory burst, and ingestion of either *Candida*⁴⁴ or coated latex beads²⁰² is associated with the appearance of the secondary granule contents in the extracellular environment. There is evidence from animal work that supernatants from PMN's stimulated with arachidonic acid or A₂₃₁₈₇ could increase the permeability of post-capillary venules in a hamster cheek pouch model²⁰³. Phagocytosis seemed a more appropriate stimulus for human studies, since the stimulus could be totally removed with the cells. The lack of permeability-producing activity in supernatants from phagocytosing PMN's suggests that the permeability factor(s) are unstable, have short half lives, or need to be generated in situ for an effect to be seen. Direct contact with endothelium, for example, may be necessary for any effect of superoxide or other oxygen radicals to become apparent. Similarly, any mast cell stimulus would only be effective if it were stable in a cell free environment. The latex beads used as a phagocytic stimulus may themselves have adsorbed out

some active factors.

In summary, therefore, the work in this chapter has not provided firm evidence of the mechanism of action of ZAS and PMN's in this system. However, the data do not suggest a major role for cyclooxygenase products in this model.

CHAPTER 6

THE EFFECTS OF HISTAMINE AND HISTAMINE ANTAGONISTS ON STIMULUS-MEDIATED COBALAMIN BINDING PROTEIN RELEASE FROM NEUTROPHILS.

(in collaboration with Neil Jackson, B Med Sci Honours student)

INTRODUCTION

The possibility that histamine antagonists might have an inhibitory effect on PMN activation arose from the observations in the last chapter on their inhibitory action on the inflammatory effects of intra-dermal complement. In normal subjects, the wheal and flare response to activated complement was significantly reduced by the prior injection of the anti-histamine drug (H_1) clemastine at high concentration.

While one obvious explanation for the inhibitory effect of clemastine in the skin was that the inflammatory effects of complement were being mediated at least in part via histamine release, an alternative explanation is that both complement fragments and clemastine were exerting their effects via PMN's. This has prompted an examination of the *in vitro* effects of histamine itself and of its antagonists on stimulus-mediated NCBP release from PMN's. Such inhibition is produced, for example, by high concentrations of anti-inflammatory agents, an effect independent of cyclo-oxygenase inhibition⁹¹.

Histamine has long been known to act via cell surface receptors, which in the last 15 years, have been considered to be of 2 classes, H_1 and H_2 ^{204, 205}. The relative distribution

of H_1 and H_2 receptors varies throughout the body, with H_1 predominating in the lung, for example, and H_2 in the gastric mucosa. Histamine activates both H_1 and H_2 receptors equally, but histamine antagonists, because of their ring substitutions show preferential binding to either H_1 or H_2 receptors²⁰⁶. This specificity is not absolute in the case of H_1 antagonists which also show some binding to H_2 receptors. H_2 antagonists show much greater specificity, with very little binding to H_1 receptors.

Physiological concentrations of histamine also appear to modulate PMN function, but reports are conflicting as to its overall effect. Inhibition by histamine of β glucuronidase release²⁰⁷, FMLP-induced superoxide production and degranulation^{208, 209}, and chemotaxis across membranes²⁰¹ have all been described. On the other hand, stimulation of cell movement²¹¹ and thromboxane production²¹² by histamine have also been reported. The nature of PMN histamine receptors is also uncertain, for both H_1 ²¹³ and H_2 ²⁰⁸ receptors have been identified.

In an attempt to clarify the situation, this study has examined the separate and combined effects of histamine and H_1 and H_2 antagonists on FMLP-induced NCBP secretion from cytochalasin B treated PMN's *in vitro*. Secretion has also been examined in PMN's from subjects after a 3 day oral course of the H_1 antagonist clemastine.

EXPERIMENTAL WORKExperiment 1 Effect of anti-histamine drugs on NCBP assay.

Method Identical aliquots of PMN sonicate were mixed with an equal volume of each drug (final concentration 10^{-4} M), histamine (10^{-3} M) or PBS as control, and the liberated NCBP assayed as before.

Results NCBP values in the presence of the drugs were always within 10% of values obtained without them. (Table 17).

Table 17 The Effect of H_1 and H_2 Antagonists on the Cobalamin Binding Protein Assay

| <u>Drug Present</u> | <u>Value of Sonicate (%)</u> | <u>S.E.M. (n=4)</u> |
|-----------------------------|------------------------------|---------------------|
| No Drug | 100.0 | - |
| Promethazine (10^{-4} M) | 92.7 | 8.2 |
| Clemastine (10^{-4} M) | 98.4 | 6.7 |
| Cimetidine (10^{-4} M) | 95.0 | 7.4 |
| Ranitidine (10^{-4} M) | 98.4 | 6.9 |
| Histamine (10^{-3} M) | 97.2 | 6.8 |

Experiment 2 Effect of clemastine on cell viability.

Method PMN's 2×10^6 /ml were suspended in clemastine 10^{-3} M for 15 minutes and their viability tested with trypan blue as described in Chapter 2.

Results Cell viability after 15 minutes incubation with clemastine 10^{-3} M (higher than concentrations used experimentally) was always >94%.

Experiment 3 Effect of H_1 and H_2 antagonists on NCBP release

by FMLP or LMW fr.

Method Injectable preparations of the H_1 antagonists clemastine and promethazine and the H_2 antagonists cimetidine and ranitidine were diluted in PBS to 10^{-3} - 10^{-7} M. 2×10^6 PMN's in 0.7ml PBS-A were incubated with 0.1ml drug for 5 minutes at 37°C . Cytochalasin B (final concentration $5 \mu\text{g/ml}$) was added and the incubation continued for a further 5 minutes. The degranulating stimulus, either FMLP (final concentration 10^{-7} M) or low MW fr (66 μl) was then added, and after 5 minutes, degranulation was terminated by centrifugation at 4°C . The supernatants were retained for NCBP assay.

Results The results of experiments involving H_1 and H_2 antagonists will be expressed as % release, where

$$\% \text{ release} = \frac{\% \text{ NCBP release with drug}}{\% \text{ NCBP release without drug}} \times \frac{100}{1} \%$$

The H_1 antagonists clemastine and promethazine both showed dose-dependent inhibition of NCBP release when stimulated by either FMLP or LMW fr (Fig 73). Promethazine was slightly more potent than clemastine at concentrations of 10^{-5} M and below, but both drugs caused complete inhibition of release at 10^{-4} M. H_2 antagonists, on the other hand, caused no significant inhibition of LMW fr-induced release at any concentration, although, when FMLP was the stimulus, there was some inhibition of release at drug concentrations of 10^{-5} M and greater (Fig 74). In all cases, however, inhibition of release was considerably less than with H_1 antagonists.

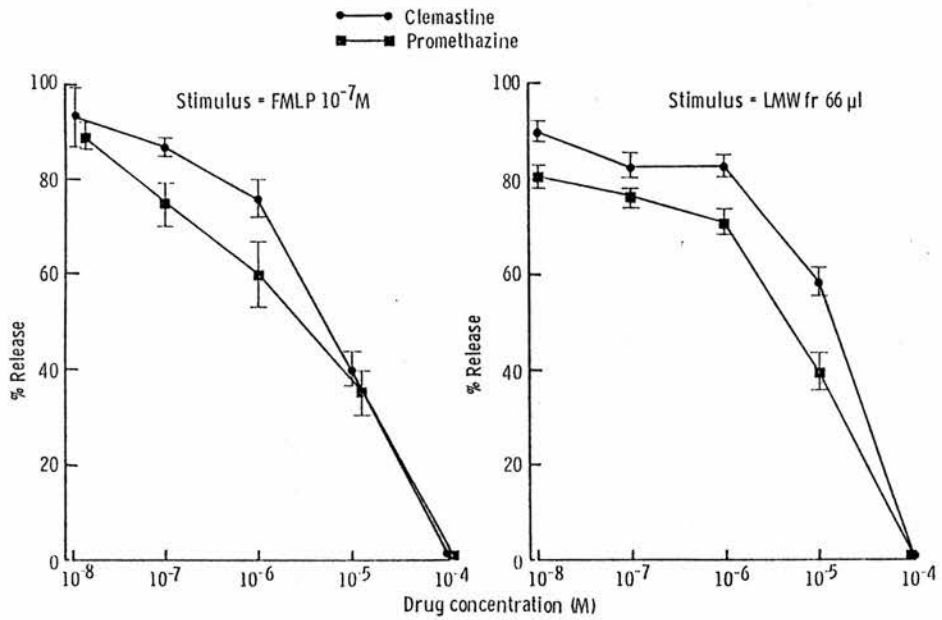


Fig 73. The effect of H₁ antagonists on NCBP release by FMLP and low molecular weight fraction.
(mean ± SEM, n = 6)

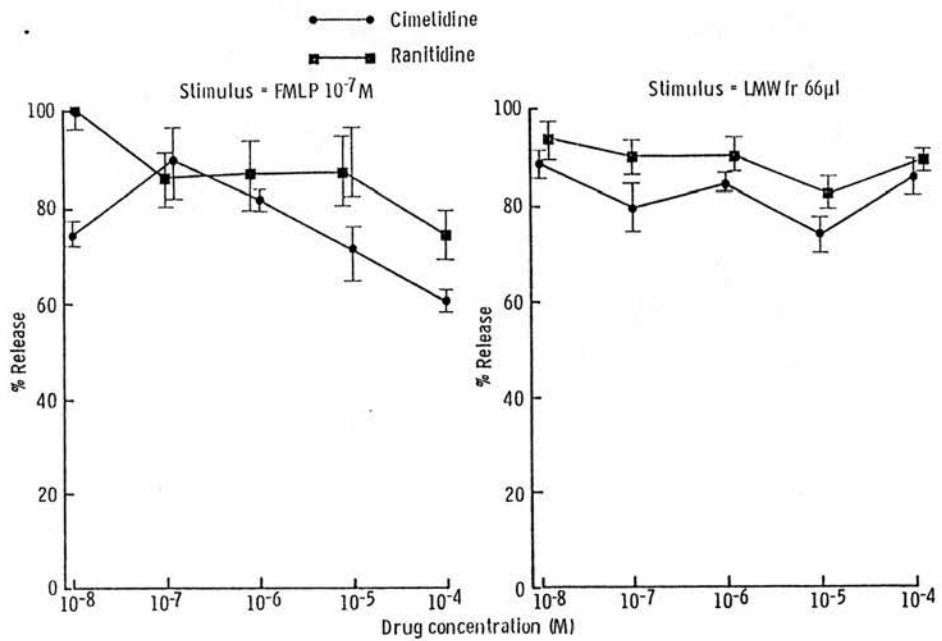


Fig 74. The effect of H₂ antagonists on NCBP release by FMLP and low molecular weight fraction.
(mean ± SEM, n = 6).

Experiment 4 Effect of stimulus concentration on H_1 antagonist-mediated inhibition of NCBP release.

Method Keeping a constant drug concentration ($10^{-6}M$), both clemastine and promethazine were used with concentrations of FMLP from $10^{-5}M$ - $10^{-9}M$, as above.

Results If the inhibitory effect of a drug can be overcome by increasing the stimulus concentration, then % release will increase with stimulus concentration, provided that the drug concentration is kept constant. When FMLP was the stimulus, neither promethazine nor clemastine at $10^{-6}M$ caused any significant change in % release between FMLP concentrations of 10^{-5} - $10^{-7}M$ (Fig 75). At $10^{-8}M$ FMLP, inhibition by promethazine actually fell, allowing 76% release, while that by clemastine greatly increased, allowing only 42% release. The reason for this difference is not apparent. When LMW fr was the stimulus, inhibition by both clemastine and promethazine was constant (but very slight) when 50 μ l or more LMW fr was added (Fig 75). With less LMW fr than this, % release by both drugs fell. In the case of LMW fr, a lower drug concentration would probably have demonstrated the effect more clearly.

These results suggest that inhibition of release by H_1 antagonists is influenced by stimulus concentration only at the lower end of the dose response curve for the stimulus, but that at higher stimulus concentrations, inhibition remains fairly constant.

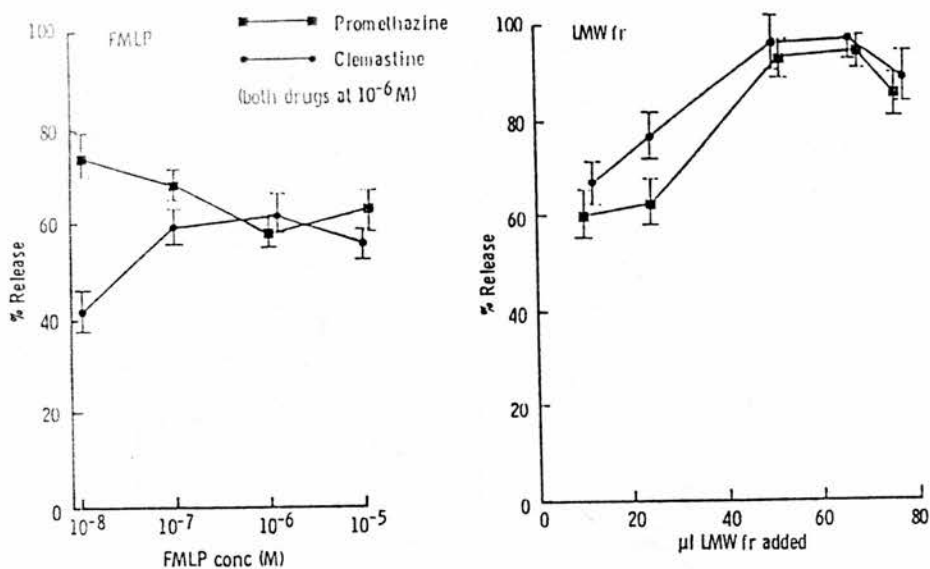


Fig 75. The effect of stimulus concentration on H_1 antagonist mediated inhibition of NCBP release.
(mean \pm SEM, n = 6).

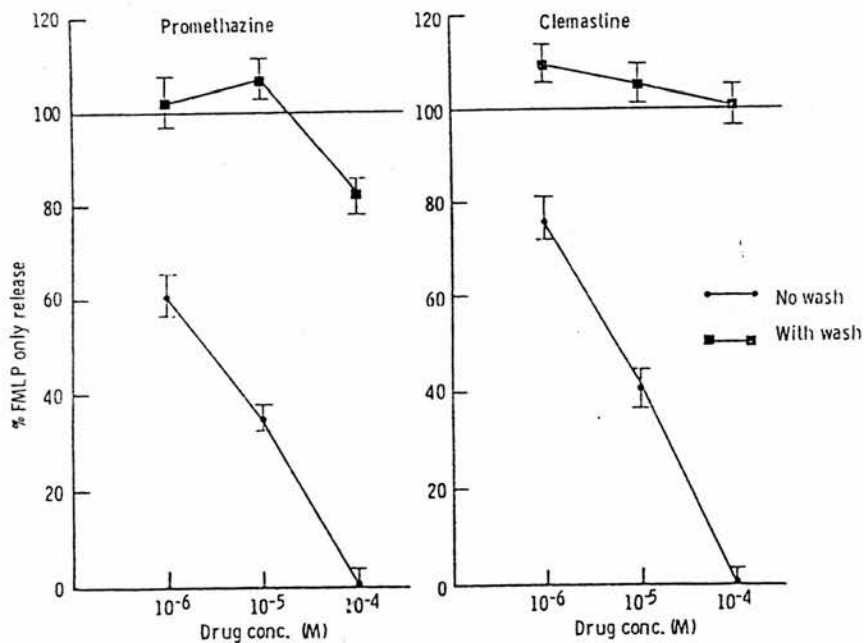


Fig 76. The effect of cell washing on H_1 antagonist mediated inhibition of NCBP release.
(mean \pm SEM, n = 6). Stimulus = FMLP 10^{-7} M.

Experiment 5 Effect of cell washing on H_1

antagonist-mediated inhibition of NCBP release.

Method PMN's 2×10^6 /ml were incubated with either clemastine or promethazine 10^{-4} - 10^{-6} M for 5 minutes at 37°C . The cells were then centrifuged at 4°C at 115g for 5 minutes and washed once by resuspending in cold PBS devoid of calcium, magnesium or albumin and centrifuging at 115g for 5 minutes. The cells were then resuspended in PBS-A and exposed to cytochalasin B and FMLP 10^{-7} M as before. After centrifugation at 4°C $50 \mu\text{l}$ supernatant was removed for NCBP assay as before. To assess cell and NCBP loss during washing, the cell pellet was resuspended and sonicated. Control cells without drug were washed and otherwise treated identically.

Results Washing the cells after incubation with either drug completely removed the inhibitory effect (Fig 76), restoring % release to 100% or greater, except for promethazine at 10^{-4} M, when % release improved from 0% to 80%.

Experiment 6 The effect of histamine on basal and FMLP-induced NCBP release.

Method Cells were exposed to either histamine alone (10^{-3} - 10^{-8} M) or histamine followed by cytochalasin B and FMLP 10^{-6} M as above.

Results Histamine alone (10^3 - 10^{-9} M) caused no release of NCBP from cytochalasin B treated PMN's. When added before FMLP, however, histamine caused dose-dependent inhibition of NCBP release (Fig 77). This was not as pronounced as the inhibition produced by H_1 antagonists (Fig 73) as, even at 10^{-3} M, 38% of the release obtained by FMLP alone was seen.

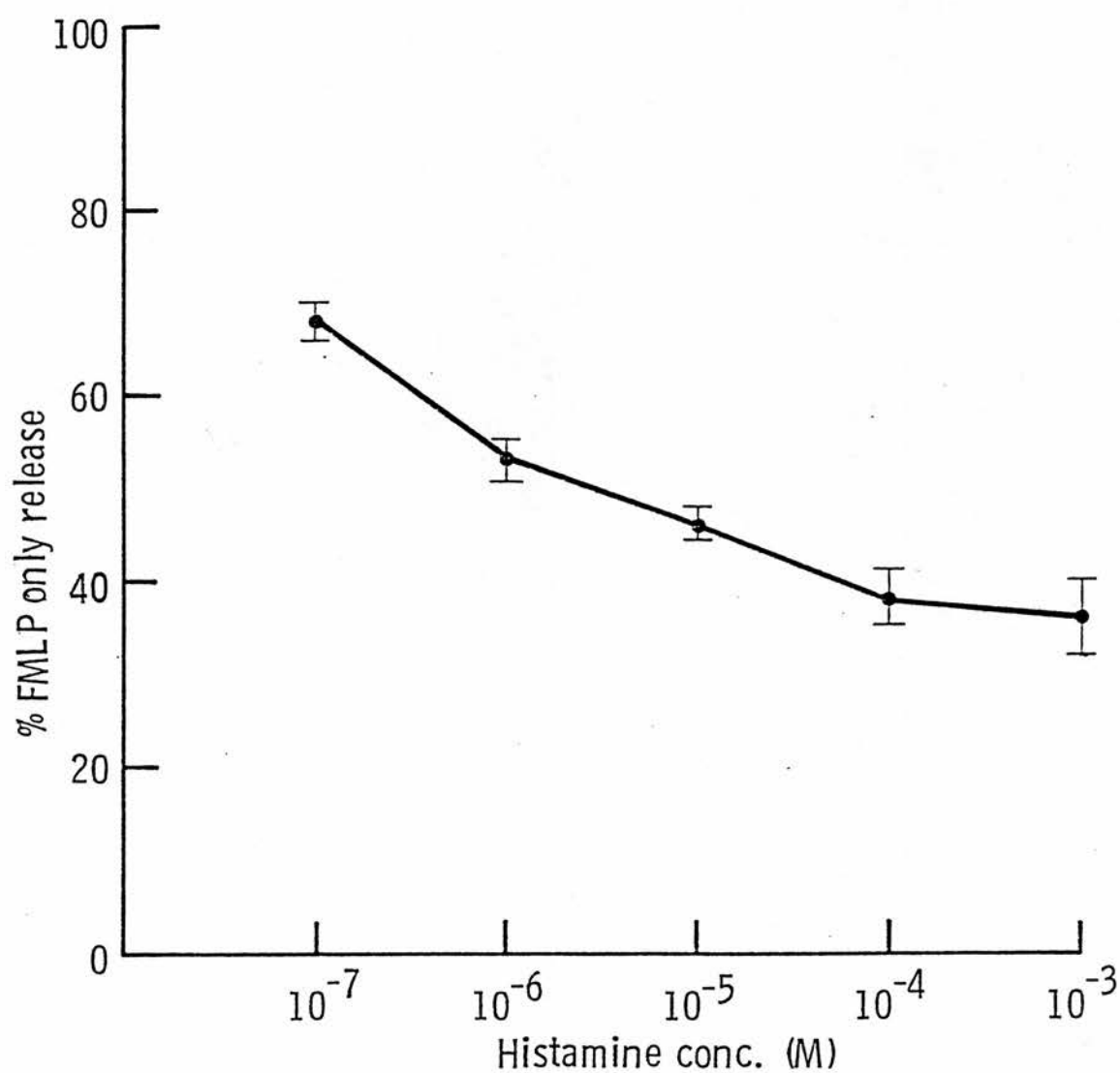


Fig 77. The effect of histamine on NCBP release from cytochalasin B treated PMN's by FMLP 10^{-6} M (mean \pm SEM, n = 6).

Experiment 7 The effects of combining H_1 and H_2 antagonists either with histamine or with each other on FMLP-induced NCBP release.

Method Cells were incubated for 5 minutes at 37°C with drug A, then, without washing, with drug B for 5 minutes. They were then exposed to cytochalasin B and FMLP 10^{-7}M as before.

The following combinations were used:-

| | <u>Drug A</u> | <u>Drug B</u> |
|-------|--|--------------------------------|
| i. | Clemastine $10^{-4} - 10^{-8}\text{M}$ | Histamine 10^{-5}M |
| ii. | Promethazine $10^{-4} - 10^{-8}\text{M}$ | Histamine 10^{-5}M |
| iii. | Histamine $10^{-4} - 10^{-8}\text{M}$ | Clemastine 10^{-6}M |
| iv. | Histamine $10^{-4} - 10^{-8}\text{M}$ | Promethazine 10^{-6}M |
| v. | Cimetidine $10^{-4} - 10^{-8}\text{M}$ | Histamine 10^{-5}M |
| vi. | Histamine $10^{-4} - 10^{-8}\text{M}$ | Cimetidine 10^{-6}M |
| vii. | Clemastine $10^{-4} - 10^{-8}\text{M}$ | Cimetidine 10^{-6}M |
| viii. | Cimetidine $10^{-4} - 10^{-8}\text{M}$ | Clemastine 10^{-6}M |

Results

A. Histamine and H_1 antagonists [(i) - (iv)]

When cells were incubated firstly with either promethazine or clemastine, and then with histamine, the inhibitory effect on FMLP-induced release was very similar to that seen when the drugs were used alone [Fig 78 (i) and (ii)]. However when histamine preceded the drugs, the inhibition seen with either drug alone or with histamine alone was abolished, so that release was restored to more than 80% of the FMLP-only value [Fig 78 (iii) and (iv)].

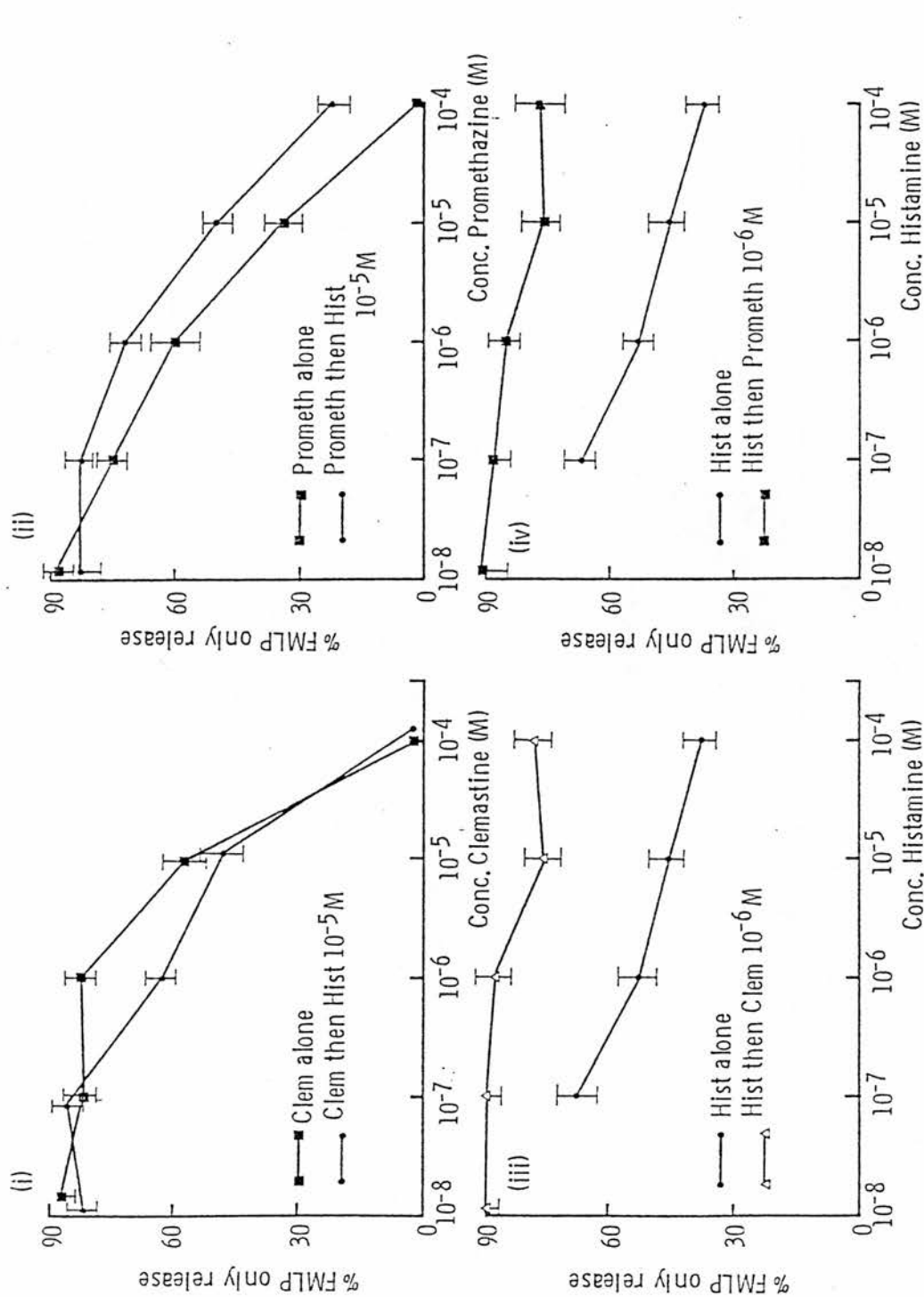


Fig 78. Effect of combining H_1 antagonists and histamine on NCBP release by FMLP 10^{-7} M (mean \pm SEM, $n = 6$).

B. Histamine and H₂ antagonists [(v) and (vi)]

When the cells were exposed to cimetidine before histamine, there was less inhibition of NCBP release at high concentrations of drug than when cimetidine was used alone [Fig 79(i)]. At drug concentrations of 10^{-6} M and below, there was no significant change in inhibitory effects. Exposing the cells to cimetidine after histamine reproduced the effect seen in Fig 78 when H₁ antagonists followed histamine, namely that the inhibitory effect of histamine alone was abolished [Fig 79(ii)].

C. H₁ and H₂ antagonists [(vii) and (viii)]

When clemastine was present first, no greater inhibition of release was produced by the subsequent addition of cimetidine [Fig 80 (i)]. The inhibitory effect of cimetidine, however, was somewhat abrogated by subsequent addition of clemastine [Fig 80 (ii)], an effect most marked at high concentrations of cimetidine.

Experiment 8 The effect of systemic clemastine on NCBP release.

Method Six healthy volunteers of both sexes, aged 21-48 took oral clemastine 1 mg b.d. (the standard therapeutic dose) for 3 days. NCBP release in response to LMW fr and FMLP was examined in PMN's prepared from each of them before and after administration of the drug. The intracellular level of NCBP in 10^5 cells (obtained by sonication) was also examined in unstimulated cells before and after administration of the drug.

Results There was no difference in intracellular levels of NCBP/ 10^5 cells before and after administration of the drug (Fig

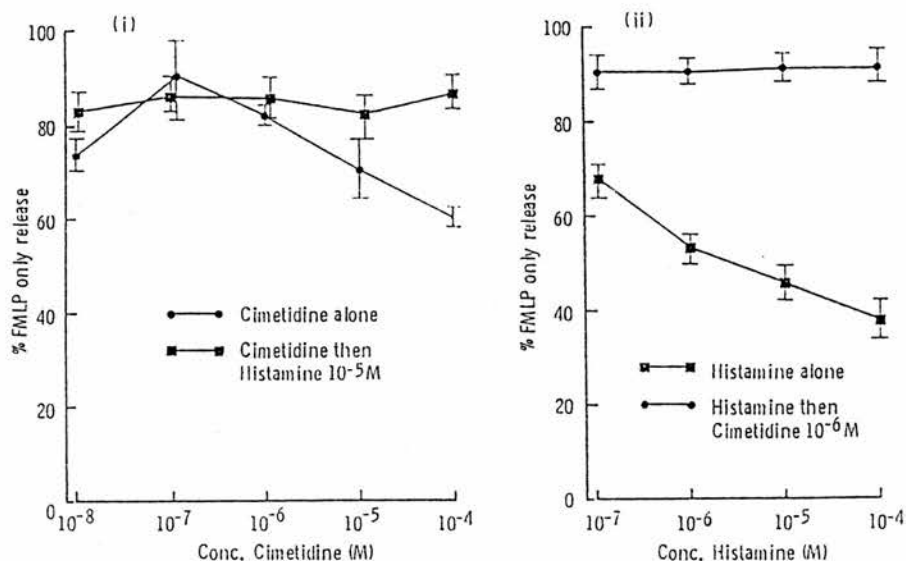


Fig 79. The effect of combining H_2 antagonists and histamine on NCBP release by FMLP $10^{-7} M$ (mean \pm SEM, $n = 6$).

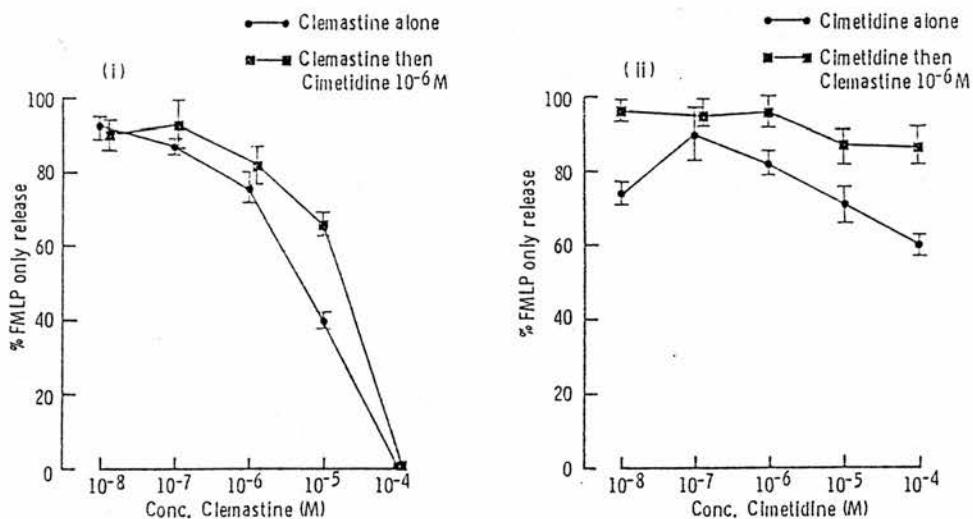


Fig 80. The effect of combining H_1 and H_2 antagonists on NCBP release by FMLP $10^{-7} M$ (mean \pm SEM, $n = 6$).

81). Similarly, NCBP release in response to 3 concentrations of either FMLP or LMW fr was not affected by oral clemastine (Fig 81).

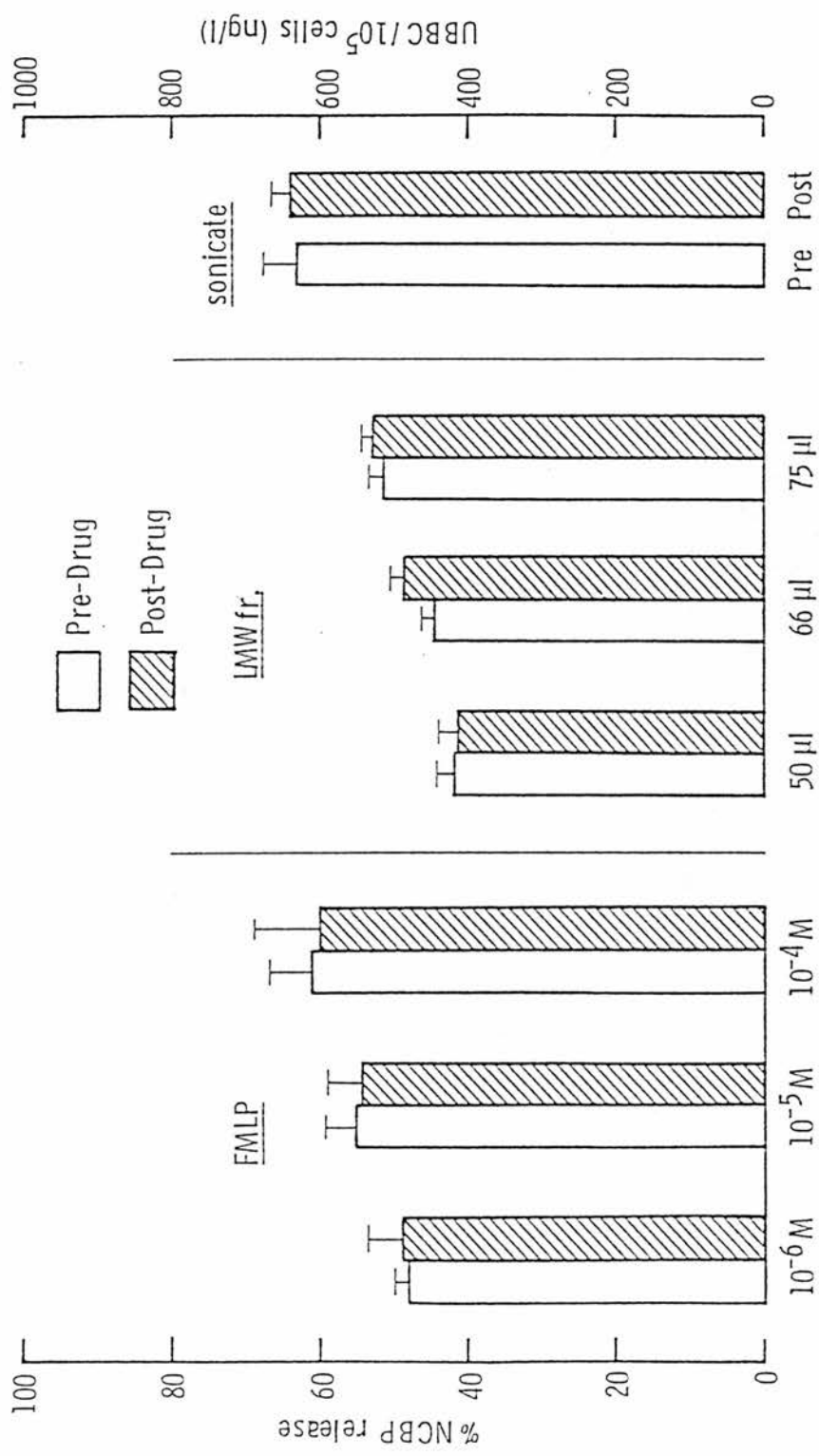


Fig 81. The effect of 3 days of oral clemastine on total intracellular NCBP and on NCBP release by FMLP and LMW fr. (mean \pm SEM, n = 6).

DISCUSSION

The effects of H_1 and H_2 antagonists on NCBP release appear to be fundamentally different. Both the H_1 antagonists tested produce dose-dependent inhibition of NCBP release in response to either FMLP or LMW fr, with complete inhibition of release at a concentration of $10^{-4}M$. This inhibition is therefore seen at drug concentrations 10-100 fold lower than those required for NSAI-mediated inhibition⁹¹. Inhibition of release by H_2 antagonists, on the other hand, is never more than 20% when LMW fr is the stimulus, and, even at a drug concentration of $10^{-4}M$ reaches only 40% when FMLP is the stimulus. Like NSAI's⁹¹, the inhibitory effect of H_1 antagonists is reversed by cell washing, suggesting reversible binding to the cell surface. Their inhibition can also be overcome to an extent by increasing the stimulus concentration, but this is much less marked than with NSAI's⁹¹, and suggests that H_1 antagonists do not work by simply competing with the stimulus for surface receptors. Other workers have similarly found no effect of these drugs on either binding or internalisation of FMLP²⁰⁸.

The effects of H_1 antagonists may best be explained by their actions on cell membranes at concentrations of $10^{-6}M$ or more, at which they have local anaesthetic type properties, inhibiting transmembrane depolarisation and calcium flux (S Hill, personal communication), events which precede NCBP release in PMN's. These effects are also seen, but to a lesser extent, with H_2 antagonists, which might explain the partial inhibition seen with these drugs at high concentrations.

In contrast to their effects *in vitro*, the inhibitory

effects of H_1 antagonists are not reproduced in PMN's from subjects taking clemastine orally. Since their effect *in vitro* is abolished by cell washing, and since PMN preparation from whole blood involves several washing steps, this lack of effect is not very surprising. It was theoretically possible that the drugs could have become incorporated into the cells during maturation and therefore remained inhibitory after cell washing, but there was no evidence that this occurred.

Another factor influencing the failure of systemic clemastine to block PMN activation is that the peak free blood level of the drug following conventional dosage is $10^{-9} M^{214}$, well below the concentrations effective *in vitro*. Considerable binding (>95%) to serum proteins is the major reason for the low level achieved (Thompson EM, personal communication).

The actions of histamine itself appear to be more complex. Although having no effects on NCBP release in resting PMN's, histamine inhibits FMLP-induced NCBP release, although with a much flatter dose-response curve than H_1 antagonists over the range tested. Such inhibition of stimulus-induced PMN responses has also been shown by other workers, with effects on degranulation^{207, 208}, superoxide and hydrogen peroxide production²⁰⁸ and chemotaxis²¹⁰. Histamine may produce these effects via an action on adenylate cyclase, since cAMP in unstimulated cells rises ten-fold in the presence of histamine $10^{-6} M^{215}$. Cyclic AMP levels in zymosan-stimulated cells are also increased by 80% by histamine $10^{-5} M^{207}$, associated with impairment of β -glucuronidase release. Such blockage of degranulation is greatly potentiated by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine²¹⁵, again suggesting

that inhibition is mediated via high levels of cAMP. The cAMP may act intracellularly to directly block protein kinase. The failure of histamine to inhibit PMN activation by the protein kinase activator phorbol myristate acetate²¹⁶ is consistent with this mode of action.

The experiments combining histamine with H_1 and H_2 antagonists attempted to determine which histamine receptors are involved in its inhibitory effects. Previous studies had reported reversal of inhibition of both cellular function and cAMP elevations by specific H_2 antagonists^{207, 208}. We have similarly found that cimetidine added before or after histamine abrogates the inhibition of release seen with histamine alone. The finding of reversal of inhibition when histamine is combined with H_1 antagonists requires an additional explanation. This may be because histamine itself, when prevented from occupying H_2 receptors by H_2 or poorly specific H_1 antagonists, begins to exert non-receptor mediated effects on the cell membrane (Hill S, personal communication). These effects tend to promote NCBP release, thus overcoming the inhibitory effects of H_1 antagonists provided the histamine is present first. Histamine added after H_1 antagonists does not alter the dose-response curves for the drugs alone. Thus histamine may have either inhibitory effects on PMN degranulation, as when no antagonists are present, or stimulatory effects, as in the presence of H_1 or H_2 antagonists.

The relevance of the above *in vitro* findings to PMN activation and inhibition remains speculative. At first glance it would appear that the concentrations of H_1 antagonists

required to inhibit PMN activation would preclude any such use clinically, being more than 1000 fold greater than the peak plasma level achievable with conventional doses. However, the same is true for NSAIDs and steroids, where 'normal' plasma levels are in the micromolar range, while levels effective *in vitro* are in the millimolar range. Despite this, gram doses of corticosteroids may be given in ARDS and other states of shock⁷⁸, during which millimolar concentrations may be reached in plasma. Local administration, for example, into the lung may also provide a means of achieving PMN inhibitory concentrations *in vivo*. Thus, as drugs with potential for reducing PMN activation, H_1 antagonists may yet find a clinical use.

The levels of histamine effective at inhibiting PMNs, on the other hand, fall well within the range present physiologically. Tissues undergoing repair have high levels of endogenous histamine²¹⁷. This may have a role in switching off the PMN activation triggered by activated complement so that PMN products do not impede the reparative processes. At the same time, PMN activation, at least by opsonised particles, releases a histaminase enzyme²¹⁸, so that a balance between PMN activation and inhibition may be struck. One situation where high histamine levels do seem to impair PMN function is in hyperimmunoglobulinaemia E where reduced chemotaxis with staphylococcal infection is seen. Interestingly the chemotactic defect can be reversed by treatment with the H_2 antagonist burimamide²¹⁹.

CHAPTER 7

INHIBITION OF NEUTROPHIL COBALAMIN BINDING PROTEIN RELEASE

AND PHAGOCYTOSIS BY EXTRACTS OF TANACETUM PARTHENIUM

(FEVERFEW) (In collaboration with Dr S Heptinstall)

INTRODUCTION

Following the observations that granule release from PMN's could be inhibited by both NSAID's⁹¹ and H₁ antagonists, it was observed that degranulation of platelets could be effectively inhibited by extracts of the herb feverfew (Tanacetum parthenium) (Heptinstall S, personal communication). This prompted examination of the effects of this herb on human PMN's.

Feverfew has been used medicinally for centuries to treat not only fever, but also such diverse disorders as migraine, arthritis, psoriasis and disordered labour and menstruation^{220, 221}. Evidence of its efficacy has till recently been largely anecdotal, but a recent controlled trial of feverfew tablets in migraine sufferers did suggest positive benefit from their use²²². At the present time, it seems likely that feverfew, in one form or another, is taken by thousands of sufferers from the above conditions²²³. Recently, there has been renewed interest in establishing the chemical nature of the active principle of feverfew and its biochemical and cellular effects.

It has long been known that an active preparation can be extracted from the plant by either water or alcohol, and

indeed a tea brewed from the leaves has been a traditional form of consumption. The literature contains little, however, on the precise mode of action of such extracts. One study has demonstrated an inhibitory effect of feverfew extract (FFE) on prostaglandin synthesis in seminal vesicles, thought not to be due to cyclooxygenase inhibition²²⁴. The possibility that FFE might influence PMN reactivity arose from studies on blood platelets. In 1981, Makheja and Bailey reported activity of a water-soluble FFE, which inhibited platelet aggregation in response to collagen, ADP, and thrombin, but not to arachidonic acid²²⁵. Thromboxane A_2 synthesis stimulated by thrombin was also inhibited, but not if an external source of arachidonic acid was provided. This study raised the possibility that FFE might be acting as an inhibitor of membrane phospholipases. Recent studies by Dr S Heptinstall and colleagues have confirmed the inhibitory actions of FFE on platelet aggregation induced by various stimuli²²⁶. Aggregation in response to adrenaline, collagen and the thromboxane A_2 (TXA₂) mimetic U46619 was abolished, and the irreversible aggregation induced by ADP and by sodium arachidonate (NaAA) was converted to a reversible response. Release of α granules, assessed by secretion of ¹⁴C-serotonin, was also inhibited by FFE, in response to ADP, adrenaline, NaAA, collagen and U46619. In contrast, secretion induced by the calcium ionophore A₂₃₁₈₇ was increased rather than decreased in the presence of FFE, and the platelet aggregation induced by A₂₃₁₈₇ was not inhibited. The effects of FFE on TXA₂ synthesis in platelets depended on the nature of the stimulus. FFE had little effect on TXA₂ synthesis induced by

NaAA, ADP or thrombin, but that induced by adrenaline was greatly reduced.

To see whether the effects of FFE on platelets could be reproduced in PMN'S, its actions on NCBP release induced by various stimuli were studied. To see whether other PMN functions could also be influenced, the effects of FFE on PMN phagocytosis of opsonised yeast have been assessed.

EXPERIMENTAL WORK

Experiment 1 Preparation of Extracts of Feverfew.

Methods

A. Chloroform-methanol extraction

Initial extracts were prepared from plants kindly supplied by Dr E W Jones, Department of Medicine, University Hospital, Nottingham. Leaves of the plant were air-dried for several weeks at room temperature, then ground to a powder in a mortar and pestle. Extraction was performed in a 1:2 mixture of chloroform and methanol (100mg powder to 5ml mixture) in a tissue homogeniser. Unextracted material was removed by centrifugation and passage through filter paper. The chloroform/methanol was blown off under N_2 and the extract resuspended in PBS. After filtration and adjustment of the pH to 7.4, the extract was stored at $-20^{\circ}C$.

B. PBS extraction

A large batch of PBS-extracted material was prepared from plants kindly supplied by Mrs Ruth Brown.

Air-dried leaves were powdered as before, then 16 grams of powder were suspended in 200 mls PBS and extracted by homogenisation in a domestic food processor for 20 minutes. Unextracted material was removed by centrifugation and filtration as before, the pH adjusted, and the extract stored at $-20^{\circ}C$ in 5ml aliquots. On thawing this extract for the first time a brown precipitate appeared, which could be removed by filtration without loss of the extract's activity. The extract was stable at $-20^{\circ}C$ for at least 3 months and could be thawed and refrozen repeatedly with only slight loss

of activity.

Result Extracts prepared in either chloroform/methanol or in PBS behaved similarly in all experiments.

Experiment 2 Effect of FFE on PMN viability.

Method Viability was tested after 15 minutes incubation with FFE using trypan blue as before.

Result Neither extract had any significant effect on cell viability which was >95% after 15 minutes.

Experiment 3 Use of Feverfew extracts as inhibitors of NCBP release.

Method The general method described in Chapter 6 was followed. The PMN's were exposed to the feverfew extracts (FFE) for 5 minutes, followed by 5 minutes with cytochalasin B (if used) and 5 minutes with the stimulus.

The stimuli used were:-

- (i) EACA ZAS
- (ii) FMLP
- (iii) Sodium Arachidonate (NaAA), previously demonstrated to be a degranulating agent for rabbit PMN's²²⁷
- (iv) A₂₃₁₈₇
- (v) Phorbol myristate acetate (PMA)

NCBP release obtained in the presence of FFE was expressed as a percentage of that obtained in the presence of stimulus plus buffer alone.

Results

(i) and (ii) EACA-ZAS and FMLP

In preliminary experiments, fixed volumes of PBS-extracted FFE greatly inhibited NCBP release from cytochalasin B treated PMN's in response to three different concentrations of EACA-ZAS or FMLP (Fig 82). The dose-response curve for FFE was almost identical for the 2 stimuli, showing significant inhibitory activity by only 20 μ l FFE with complete inhibition by 200 μ l (Fig 83).

(iii) Sodium arachidonate (NaAA)

Preliminary experiments established significant release of NCBP by 0.25 mM AA within 5 minutes whether cytochalasin B was present or not (Fig 84a). Release by 0.1mM AA was little enhanced by the addition of CB, and the increase produced by it with 0.25 mM AA was much less than that seen with either FMLP or EACA-ZAS (Chapters 2 and 3). Release by 0.25 mM AA, either with or without CB, was inhibited in a dose-dependent manner by either PBS-extracted or chloroform/methanol-extracted FFE (Fig 84b). The shape of the dose-response curve differed to that seen when either FMLP or EACA-ZAS was the stimulus (Fig 83), with much less inhibition of release by low concentrations of FFE.

(iv) A_{23187}

Significant NCBP release (45-50%) was demonstrated in Chapter 3 by 2 μ M A_{23187} . Release by this stimulus in the presence of chloroform-methanol-extracted FFE demonstrated, with small amounts of FFE, a steady rise in NCBP release (Fig 85), reaching 140% of control values with 50-70 μ l FFE. This enhancement of release was less marked at higher FFE

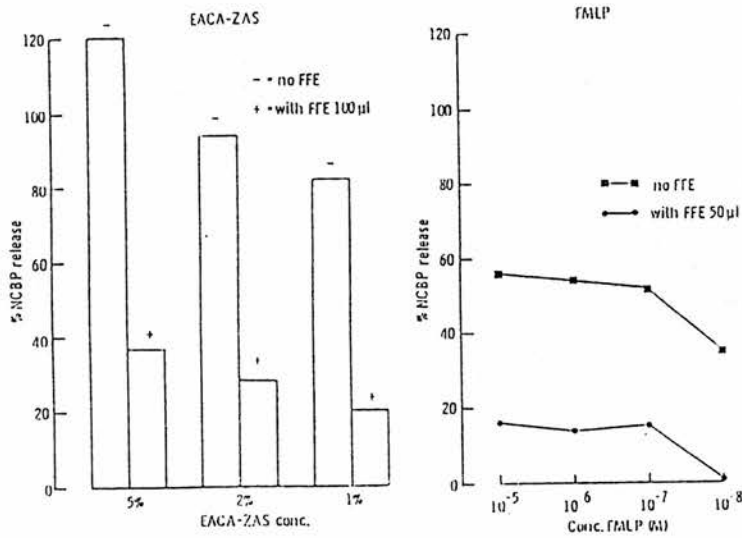


Fig 82. NCBP release from cytochalasin B treated PMN's by EACA-ZAS and FMLP without and with constant volumes of FFE (n = 2).

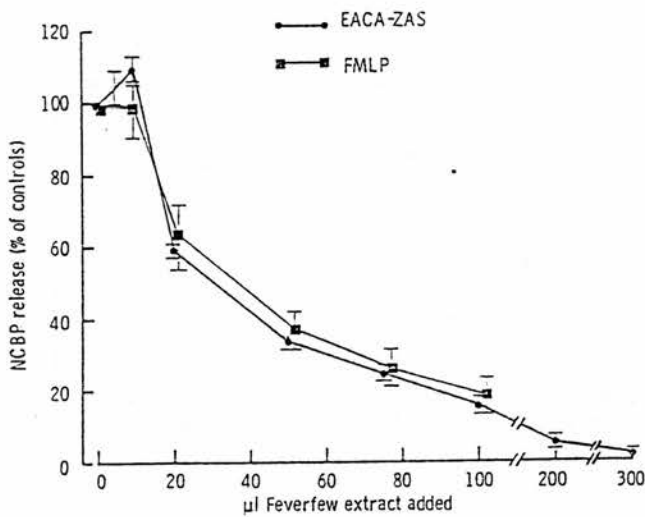


Fig 83. The effect of PBS extracted FFE on NCBP release from cytochalasin B treated PMN's by 5% EACA-ZAS and 10^{-5} M FMLP. (mean \pm SEM, n = 4).

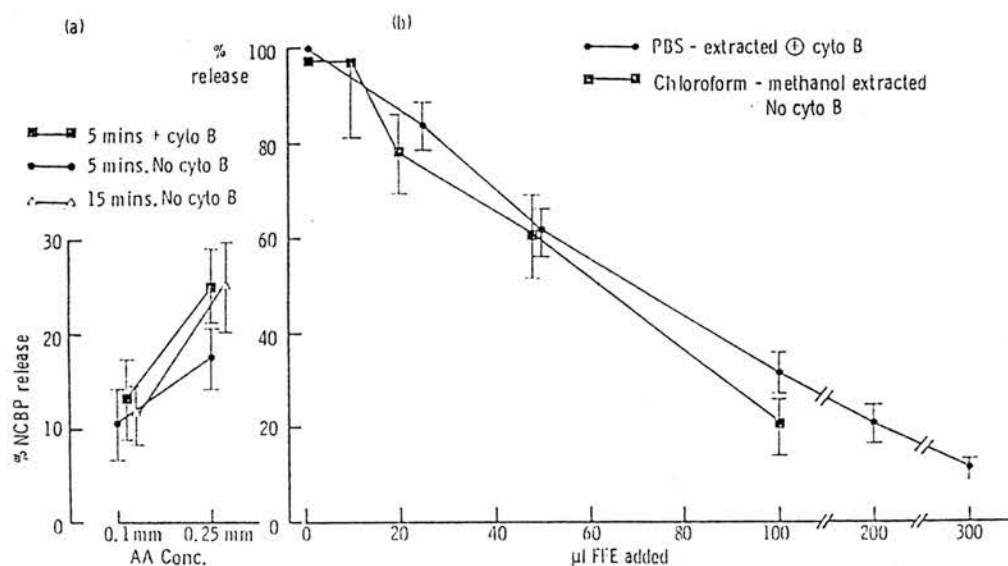


Fig 84. NCBP release by arachidonic acid and % release by PBS- and chloroform-methanol extracted FFE with 0.25mMAA with and without cytochalasin B (mean \pm SEM, n = 4).

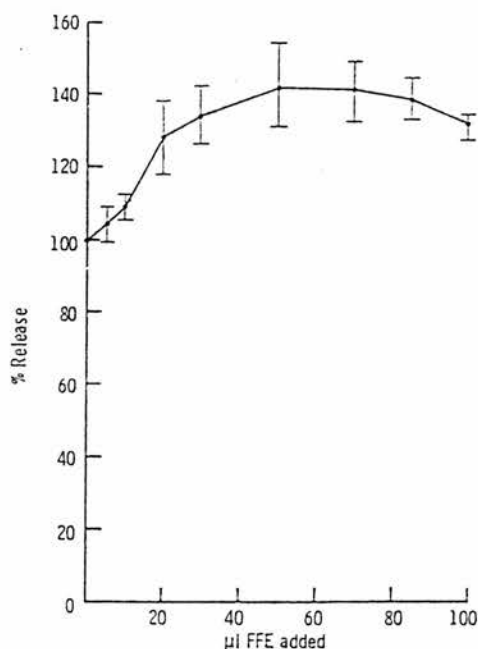


Fig 85. The effect of chloroform-methanol extracted FFE on NCBP release from non-cytochalasin B treated PMN's by 2µM A₂₃₁₈₇. (mean \pm SEM, n = 4).

concentrations, but with up to 100 μ l FFE, no inhibition of release was produced. This effect is therefore fundamentally different to that seen with other stimuli.

(v) Phorbol myristate acetate (PMA)

Fig 86 shows the dose response curve for NCBP release by PMA in the presence and absence of FFE (100 μ l PBS-extracted). At all concentrations of PMA, the addition of FFE significantly inhibited NCBP release. At a constant PMA concentration of 500 ng/ml, addition of 30 μ l or more of FFE caused significant inhibition of release (Fig 87).

Experiment 4 The effect of timing of addition and cell washing on the inhibitory effects of FFE.

Method To ensure that FFE was not merely inhibiting the actions of cytochalasin B, comparison was made of inhibition when the order of addition was:-

- a) FFE \longrightarrow CB \longrightarrow stimulus (either FMLP or LMW fr)
- b) CB \longrightarrow FFE \longrightarrow stimulus
- c) CB \longrightarrow FFE/Stimulus together

To see whether the effect of FFE could be removed by washing, cells were incubated in FFE for 5 minutes as before, then spun at 115g for 5 minutes. They were then washed once by resuspension in cold PBS and centrifugation at 115g for 5 minutes, then exposed to cytochalasin B and stimulus as before.

Results Fig 88 shows that the effect of FFE was identical whether it was added before the CB (bar 2) or after the CB (bar 4). To achieve maximal inhibition, it appeared necessary to add the FFE before the stimulus (bar 2), for when it was

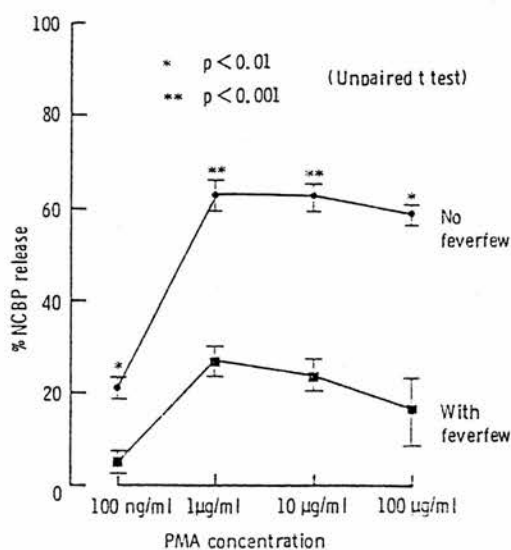


Fig 86. NCBP release from cytochalasin B treated PMN's in the presence and absence of 100µl PBS-extracted feverfew by phorbol myristate acetate (PMA). (mean \pm SEM, n = 4).

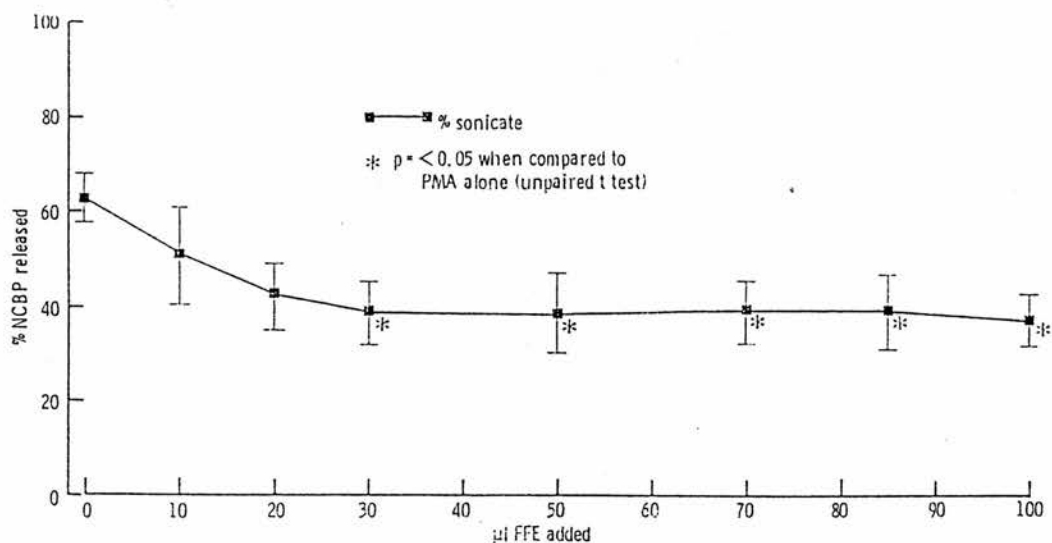


Fig 87. NCBP release from cytochalasin B treated PMN's by phorbol myristate acetate (500ng/ml) in the presence of different volumes of PBS-extracted FFE (mean \pm SEM, n = 4).

added at the same time as the stimulus (bar 5), NCBP release approached, although did not reach, the level produced by stimulus alone (bar 1). The effect of washing the cells after incubation with FFE seemed to depend to an extent on the stimulus. For LMW fr, washing removed very little of the inhibitory effect (bar 3), but when FMLP was the stimulus, washing removed about a third of the inhibition seen (bar 3).

Experiment 5 Separation of FFE into low molecular weight (<30,000 Daltons) and high molecular weight (>30,000 Daltons) fractions.

Methods 1ml aliquots of PBS-extracted FFE were applied to the 30,000 MW partition membrane as described in Chapter 2 and centrifuged at 2000g at room temperature for 30 minutes. The resulting fractions were tested for inhibitory activity as above.

Results The inhibitory fraction of FFE, appeared to be, as expected, of low molecular weight, for while the high MW fraction produced no inhibition of FMLP-induced NCBP release, the dose-response curve for the low MW fraction closely resembled that for whole FFE (Fig 89).

Experiment 6 The effect of FFE on PMN phagocytosis of *Candida guilliermondii*.

Method *C. guilliermondii* cultured in glucose broth were sonicated for 30 seconds to break up clumps, and opsonised by incubation with fresh human serum for 60 minutes at 37°C on a rotator. After centrifugation, the *Candida* were resuspended at a concentration of 4×10^7 /ml in tissue culture medium TC

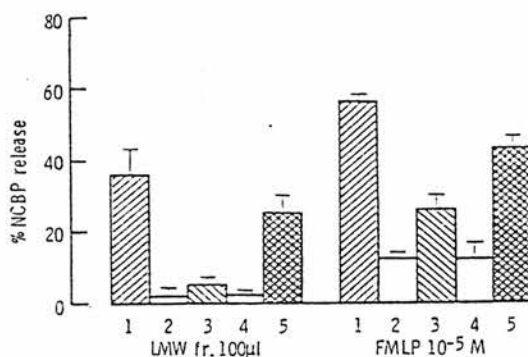


Fig 88. Effect of washing and time of addition on the effect of 50 μl PBS-extracted FFE on NCBP release from cytochalasin B-treated PMN's by 100 μl LMW fr. or FMLP 10⁻⁵ M (mean ± SEM, n = 4).

- 1 CB + stimulus only
- 2 FFE → CB → stimulus
- 3 FFE → wash → CB → stimulus
- 4 CB → FFE → stimulus
- 5 CB → FFE/stimulus together

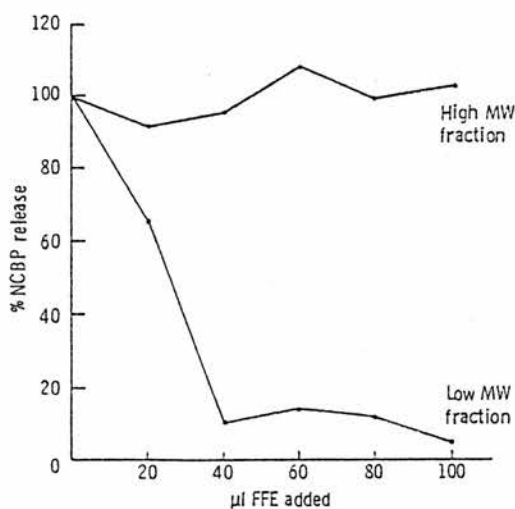


Fig 89. NCBP release from cytochalasin B treated PMN's by FMLP 10⁻⁵ M in the presence of the high and low MW fractions of FFE (n = 2).

199. The Candida were incubated with PMN's at a ratio of 4.5 Candida : 1 cell for 45 minutes at 37°C to allow phagocytosis. This ratio and incubation period have previously been shown to be optimal for phagocytosis to occur¹⁸⁶. The reaction was stopped by the addition of cold 6mM EDTA and centrifugation at 4°C . Cytospin preparations were made and stained with Leishman stain. Three replicate coded slides were studied microscopically for each experimental condition. Two hundred PMN's on each slide were examined, and the number of Candida in each cell counted. The data were then analysed in 2 ways:

1. the percentage of PMN's containing one or more Candida was calculated and termed 'percentage phagocytosis'¹⁸⁶.

2. the number of Candida in each cell was counted and a distribution histogram constructed of cells containing :

0 Candida

1-2 "

3-5 "

>5 "

This allowed for an effect of FFE on the number of Candida which each cell could take up, rather than simply making a crude assessment of whether the cell could phagocytose any yeasts at all.

Results Incubation of phagocytosing PMN's with FFE

significantly increased the number of cells which fail to ingest any Candida at all (Fig 90). In addition, cells which were actively phagocytosing ingested significantly fewer Candida than normal in the same time period when FFE was present, shifting the distribution curve for number of Candida .

ingested to the left (Fig 91).

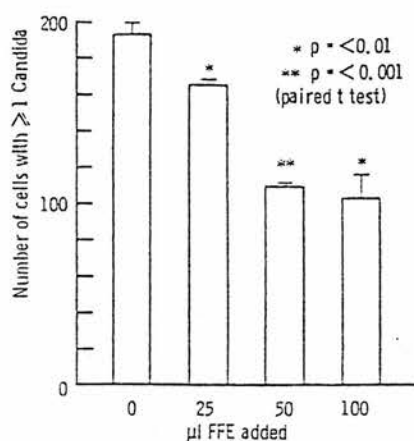


Fig 90. Effect of PBS-extracted FFE on phagocytosis of *Candida guilliermondii*, showing the number of cells which have phagocytosed ≥ 1 *Candida*. Mean ± SEM, n = 3, with each having 3 replicates.

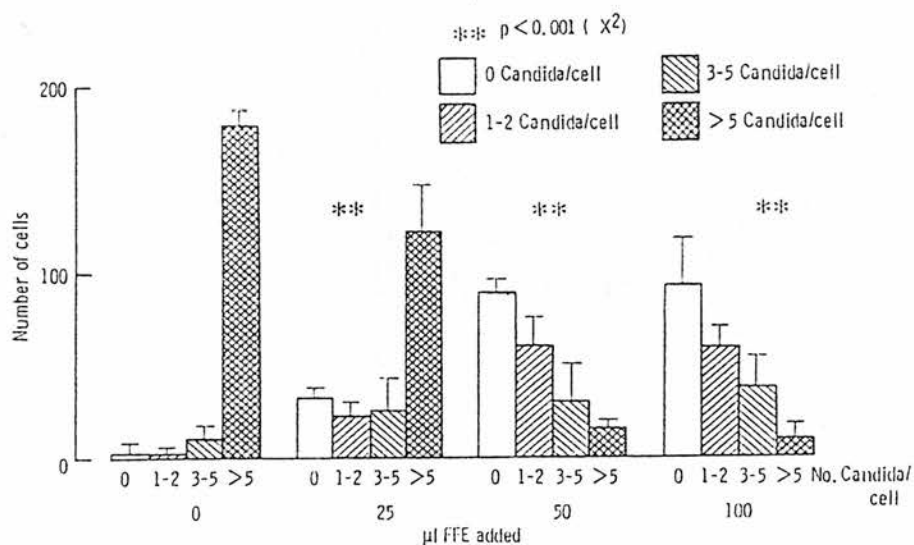


Fig 91. The effect of FFE on the number of *Candida* ingested by PMN's (mean ± SEM, n = 3, each with 3 replicates).

DISCUSSION

Both water-soluble and lipid-soluble extracts of FFE inhibited NCBP release from activated PMN's, while water-soluble preparations could also inhibit phagocytosis. Inhibition of degranulation was most marked with stimuli operating through cell surface receptors, such as FMLP and EACA-ZAS, but release in response to arachidonic acid was also blocked. This argues against the suggestion that feverfew operates as a phospholipase inhibitor²²⁵, as the exogenous arachidonic acid provided would be able to bypass the blockage of release of cellular arachidonic acid from membrane phospholipids. Observations in platelets also suggest that inhibition of degranulation or aggregation by FFE can occur either without inhibition of thromboxane synthesis, or under conditions where thromboxane synthesis is prevented by aspirin²²⁶. Thus another mechanism of action must be postulated.

The effects of FFE, unlike those of non-steroidal anti-inflammatory drugs, cannot be reversed by washing the cells, suggesting a mode of action other than blockade of stimulus-receptor binding. Occupation of cell surface receptors by a stimulus triggers activation of two separate stimulus-response coupling mechanisms within the cell. These are firstly calcium mobilisation²⁵ and secondly activation of protein kinase C by the formation of diacyl glycerol from membrane lipids²⁷ (see Chapter 1, Section A).

Two pieces of evidence suggest that FFE has little effect on calcium mobilisation. Firstly, PMN and platelet degranulation stimulated by the calcium ionophore A₂₃₁₈₇ is

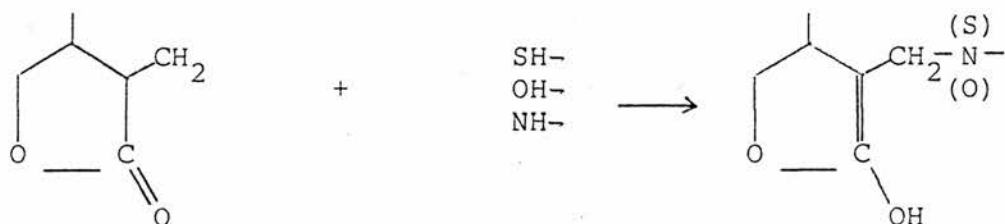
not inhibited by FFE²²⁶, and is in fact somewhat enhanced. Secondly, FFE is able to block platelet thromboxane synthesis in response to adrenaline, a stimulus which differs from other platelet activators in causing no increase in the concentration of free calcium within the platelets²²⁸. Thus an alternative site of action for FFE must be found, which may prove to be at sites on the activation pathway for protein kinase C, the pathway probably involved in adrenaline-mediated activation of platelets. A further observation which suggests that the pathway to protein kinase C activation is at least partially blocked by FFE is that PMN degranulation by PMA, which activates protein kinase C directly, is rather less inhibited by FFE than is degranulation in response to stimuli which act via surface receptors. However, FFE does inhibit PMA-induced secretion to a significant extent, suggesting that blockade of protein kinase C activation is not the only site of action of FFE. The microfilament/microtubule system important in both phagocytosis and secretion may be one possible site.

Phagocytic processes in PMN's are susceptible to inhibition by a number of compounds of plant origin. The fungal metabolite cytochalasin B, despite its enhancement of degranulation⁴⁹, is inhibitory by virtue of its ability to block the polymerisation of cytoskeletal actin²²⁹. Colchicine, from the plant *Colchium autumnale*, also blocks phagocytosis, this time by inhibiting the cytoplasmic microtubule system important in cytoskeletal orientation and stability²³⁰. Unlike cytochalasin B, colchicine also inhibits stimulus-mediated granule release, but appears to be much less

potent than feverfew. Release of β glucuronidase by opsonised zymosan particles was reduced only by 40% by colchicine at a concentration of $10^{-5} M^{231}$.

The exact chemical nature of the active principle(s) in FFE has not yet been identified. The plant contains a group of sesquiterpene lactones which are lipophilic. The main lactone in FFE is parthenolide (MW = 248 daltons), from which the plant takes its name. Parthenolide has similar effects to whole FFE on platelets, (Dr S Heptinstall, personal communication) and other fractions active in platelet inhibition have also been identified. Like parthenolide, they all contain a chemical grouping which can undergo interaction with SH-, NH- or OH- groups.

Thus:



known as the Michael addition.

The principal source of SH-groups in platelets is glutathione. Measurable levels of glutathione SH groups in resting platelets fall with time after addition of FFE, an effect which can be prevented by cysteine (containing -SH) but not serine or glycine (Dr S Heptinstall, personal communication). Cysteine can also reverse some of the inhibition of platelet aggregation and degranulation produced by FFE. It is not yet known whether similar involvement of SH groups is seen in the effects of FFE on PMN's, but it may be relevant that the assembly of microtubules required for both

granule secretion and phagocytosis requires conversion of reduced glutathione (glutathione - SH) to the oxidised form G-S-S-G²³².

It remains to be seen whether PMN's from subjects taking oral feverfew show the same inhibition of degranulation and phagocytosis as is seen *in vitro*. While blockade of granule release in platelets and PMN's might be beneficial to an extent in migraine and rheumatoid arthritis, the effects on phagocytosis may prove to be an undesirable side effect. If as is suggested²²³ further clinical trials of feverfew will be undertaken, then studies of PMN function should be an integral part of such trials.

CHAPTER 8

CONCLUSIONS, AND IMPLICATIONS OF NEUTROPHIL SECRETION IN THE INFLAMMATORY RESPONSE

The studies described here have demonstrated the importance of C_5 as a source of degranulating activity for PMN's, have examined the inhibition of such degranulation by H_1 antagonists and feverfew extracts, and have attempted to investigate the ways in which C_5 fragment-induced PMN activation might produce microvascular changes *in vivo*.

What are the implications of these findings in the understanding of inflammatory and immune processes?

C_5 fragments appear to be the main fluid phase complement-derived chemotactic stimulus for PMN's but it is difficult to assess their importance in the anti-bacterial role of the cells. Complement activation leads to coating of bacteria by C_{3b} , for which PMN's have receptors, but C_5 fragments may also be important in bringing PMN's and bacteria together. Human C_5 may be deficient in either quantity or function. One family in whom C_5 was less than 0.05% of normal in 4 of 5 siblings, experienced recurrent infections with the intracellular pathogens *Neisseria meningitidis* and *N. gonorrhoeae*²³³. As expected, chemotactic activity could not be generated by zymosan from their sera, although no defects of phagocytosis or killing were present. The CH_{50} assay showed no detectable haemolytic activity in the C_5 deficient individuals, while the alternative complement pathway haemolytic assay showed a delayed endpoint corrected by the

addition of C_5 . No C_5 inhibitor was present. Neisserial infections are well recognized in individuals with defects of any of the membrane attack complex components²³³. How protection from other bacteria is achieved is not certain, for the maximum concentration of C_{5a} which could be generated from serum containing less than 0.05% of normal C_5 is 5 ng/ml, only one tenth of the minimum concentration detectable by chemotaxis. The direct chemotactic action of bacterial peptides such as FMLP²¹ may largely compensate.

Functional defects of immunologically normal C_5 have been described in a family where the defect was present in 15 relatives¹⁴⁷. The index case was a child who had repeated local and systemic infections with various Gram-negative bacteria and coagulase positive staphylococci. The patient's serum showed inability to enhance chemotaxis towards, or phagocytosis of *E coli*, *Staph aureus* or yeasts, a defect correctable by the addition of normal serum or C_5 , but not by C_3 . Phagocytosis of pneumococci, which is independent of C_5 , was normal. C_5 in the serum was normal by both IEP and immunodiffusion.

It is of interest that quantitative C_5 deficiency and functional impairment of C_5 should render individuals susceptible to different groups of micro-organisms. It may be that the abnormal C_5 actually blocks the binding of any normal C_5 , or of other complement components, thus compounding the defect and increasing the range of bacteria which cannot be ingested. Further detailed study of the C_{5a} molecule in such families could reveal much about the nature of C_{5a} -neutrophil binding and activation.

An inhibitor of C_{5a} -induced chemotactic activity has also been described in patients with SLE, and has been implicated in the increased susceptibility to pyogenic infections seen in that condition²³⁴. The inhibitor described did not inhibit chemotaxis in response to other stimuli, and, oddly, did not impair C_{5a} -mediated aggregation or degranulation. It appears to be quite different to the chemotactic factor inhibitor described by Berenberg and Ward⁶⁸. It thus appears that C_5 fragment-mediated PMN activation is physiologically important in anti-bacterial defence.

The *in vivo* studies performed here also suggest a key role for activated PMN's in the production of complement-induced permeability changes in small vessels. However, not every situation in which PMN's are stimulated leads to such permeability changes and tissue injury. For example, the well-described dialysis leucopenia⁹³, thought to be due to complement activation and pulmonary PMN sequestration, has never been reported to cause clinical features of ARDS. Studies in sheep infused with zymosan activated plasma also report marked PMN accumulation in alveoli, but although there is early endothelial injury, this has recovered by 4 hours, even though PMN's remain in the pulmonary micro-circulation²³⁵. The pulmonary damage following infusion of cobra venom factor into rats has also been shown to be transient²³⁶. Experiments using cultured endothelial cells from bovine pulmonary arteries in Boyden chambers have demonstrated that PMN's can migrate across endothelial layers in response to activated complement without

damage to the endothelium, as demonstrated by light and electron microscopy. The PMN's appear to remain in close proximity to the endothelium at all times such that no leak of radiolabelled water or sucrose is seen²³⁷. In contrast, the addition of histamine to the preparation causes focal dilatations in junctions between endothelial cells, with leakage of radiolabelled solutes²³⁸. Thus additional factors must dictate whether permeability changes result from contact between activated PMN'S and endothelium. In health there is a large pool of margined PMN's, particularly in the lungs, yet no damage to lung tissue results. Traditional concepts of complement-induced lung injury have assumed that the important event is the activation of PMN's, with the endothelium being damaged almost as an 'innocent bystander'. However, it now appears that the endothelium plays a more active role in influencing the outcome of such PMN activation than previously thought. It has been suggested that direct damage to or other alterations in endothelial cells themselves must take place before activated PMN's can further impair their integrity²³⁹. For example PMN adherence to endothelium can be increased by exposure of the endothelial layer to endotoxin²³⁹, viruses²⁴⁰ or hyperbaric oxygen²⁴¹. Such damage may also expose endothelial receptors for Fc and C_{3b}²⁴², thus providing a mechanism for cell to cell adhesion with PMN's coated with immune complexes and complement. Normal intact endothelium may actually limit PMN contact with itself, by promoting PMN emigration through the vessels into the tissues. PMN's in Boyden chambers, for example, move much more rapidly through into pulmonary artery implants when the endothelial layer is

intact²³⁹.

It may be therefore, that to produce permeability changes and tissue damage a stimulus has to not only activate PMN's but also damage endothelium directly, and that stimuli which affect only PMN's will cause only mild and transient endothelial damage. Circulating factors such as fibrinogen²⁴³ and fibronectin²⁴⁴ also alter PMN adherence to endothelium, as do PMN factors themselves, particularly lactoferrin⁵². Relatively little is known about endothelial activation and behaviour, but further study of PMN-endothelial interaction may fill in many of the gaps in knowledge of the inflammatory processes.

Where these processes are damaging to tissues it may be of advantage to block PMN responsiveness, at least partially. High concentrations of anti-inflammatory drugs can do so *in vitro*, and now H₁ antagonists and feverfew extracts can be added to the list of drugs active in this way. The difficulty in translating *in vitro* activity into clinical effect is well demonstrated by the observation that conventional doses of hydrocortisone and anti-inflammatory agents completely failed to block skin responses to activated complement, perhaps because plasma levels of the drugs were too low to inhibit PMN activation. Similarly, degranulation of PMN's from subjects taking clemastine orally was normal, again because of failure to achieve high enough concentrations of the drug in plasma. The problem of achieving suitably high drug concentrations *in vivo* could perhaps be overcome by local administration to affected sites, for example via a nebuliser to the lungs or directly into inflamed joints.

As well as modifying endothelial function, secretory products from activated PMN's can influence the behaviour of other cell types, notably monocytes and macrophages, and thereby the activity of T and B lymphocytes and granulocyte precursors. Impaired lymphocyte reactivity and absent skin responses to various T and B cell mitogens have been reported in patients with peripheral blood PMN counts of $>15 \times 10^9/l$ from whatever cause (excluding chronic granulocytic leukaemia)²⁴⁵. Other possible causes of this anergy were excluded as far as possible, and normal responses returned when the PMN count fell. Of course the underlying causes of the neutrophilia could have produced anergy by independent means, but it remains an interesting speculation that factors from PMN's themselves could have been responsible.

The monocyte/macrophage factor interleukin-1 (IL-1) appears to link many of the diverse effects of infection and inflammation, causing neutrophilia, fever, breakdown of muscle protein, synthesis of collagen from fibroblasts, and release of acute phase proteins from the liver as well as activation of T and B lymphocytes²⁴⁶. The neutrophilia is primarily due to release of young PMN's from the bone marrow. Factors from PMN's undergoing phagocytosis or activation by soluble stimuli may be able to modulate IL-1 synthesis and thus influence many aspects of inflammatory and immune responses. This could certainly produce anergy and may be relevant in the T cell unresponsiveness of Hodgkin's disease, in which PMN's appear to be hyperactive, with high neutrophil alkaline phosphatase scores²⁴⁷, and increased superoxide production^{248, 249}. In addition, pulmonary fibrotic disease may be partly due to

activated PMN's causing increased IL-1 synthesis by pulmonary macrophages, with subsequent stimulation of collagen production²⁵⁰. A further monocyte product inhibited by PMN secretions is the colony stimulating factor required for the growth of myeloid colonies in culture⁵⁵. This feedback control of granulocyte numbers appears to involve lactoferrin⁵⁷, and may be defective in chronic granulocytic leukaemia¹⁹³.

It can thus be seen that the PMN has important secretory functions. Release of granule contents, oxidation products and lipid mediators all play a part in the generation of the features of inflammation. The studies described here have demonstrated *in vitro* and *in vivo* models suitable for the assessment of possible inhibitors of these often undesirable responses.

APPENDIX 1SUPPLIERS OF MATERIALS1. Laboratory equipment

Blood cell counter and Isoton II: - Coulter DN, Coulter,
Harpenden.

Centrifuges: - Damon IEC Centra 7

- 4⁰ centrifuge - Beckman TJ6

- Microcentrifuge - MSE.

Cell sonicator: - Rapidis 'S' model - Ultrasonics Ltd,
Shipley.

Y counter: - Intertechnique CG4200.

Spectrophotometer: - Pye Unicam SP1800.

Cytospin: - Shandon-Elliott.

Filter systems: - 0.8 μ - Millipore

0.2 μ - Millipore.

Micropartition membrane and holders: - Amicon Corporation.

LP₃ polypropylene tubes: - Luckham's.

Universal containers: - Sterilin.

1ml syringes: - Gillette (Sabre).

27 gauge needles: - Monoject.

Skin calipers: - Mitutoyo, Japan.

Serum harvester: - Glasrock Products Inc.

2. Chemicals

Agarose: - Miles Serevac.

Monopoly resolving medium: - Flow Laboratories, Irvine.

Lymphocyte separation medium: - Flow Laboratories, Irvine.

Phosphate buffered saline tablets:- Flow Laboratories, Irvine.

Earl's balanced salt solution: - Flow Laboratories, Irvine.

$^{57}\text{CoB}_{12}$ (high specific activity): - Radiochemicals.
Centre, Amersham.

Dextran - (Lomodex 70): - Fisons Ltd, Loughborough.

Candida guilliermondii: - Public Health Laboratory,
Nottingham.

Diffquik: - Merz and Dade.

Epsilon amino caproic acid: - BDH.

Ethylene diamine tetraacetic acid (EDTA): - BDH.

Ethylene-bis-(oxy-ethylene-nitrile)
tetraacetic acid (EGTA): - BDH.

Calcium chloride: - BDH.

Magnesium chloride: - BDH.

Bovine haemoglobin: - BDH.

Sodium azide: - BDH.

Glycine: - BDH.

Ammonium chloride: - BDH.

Phorbol myristate acetate: - Sigma.

Latex beads: - Sigma.

Dimethyl sulphoxide: - Sigma.

Formyl-methionyl-leucyl-phenylalanine: - Sigma.

A $^{231}\text{87}$: - Sigma.

Sephadex G200: - Sigma.

Cyanogen bromide activated sepharose 4B: - Sigma.

Bovine serum albumin: - Sigma.

Zymosan: - Sigma.

Coomassie blue: - Sigma.

Activated charcoal: - Sigma.

Ethanolamine: - Sigma.

Trypan blue: - Sigma.

Cytochalasin B: - Sigma.

Arachidonic Acid:

- Sigma.

3. Drugs

Clemastine: - Sandos.

Histamine: - McCarthy's Ltd.

Cimetidine: - Smith, Klein & French.

BW 755C: - Boots Ltd.

Prostaglandin E₂: - Upjohn Ltd.

Hydrocortisone:

- Hydrocortisone sodium succinate BP - Boots Ltd.

Ibuprofen: - Boots Ltd.

Aspirin: - Aspirin tablets BP.

4. Antisera

Anti-H IgG - Goat anti-human IgG (gamma chain):

- Kent Laboratories. Lot No 1010 AM3.

Anti-H C3 (1) - Rabbit anti-human C3 (C3, C3c, C3d):

- Blood Transfusion Service, Bristol.

Anti-H C3 (2) - Rabbit anti-human C3 (C3d):

Dako Laboratories. Lot No A063 - 010D.

Anti-H C3 (3) - Goat anti-human C3 (C3, C3c):

Kent Laboratories. Lot No 103053.

Anti-H C3 (4) - Sheep anti-human C3 (C3, C3a, C3c):

Seward Laboratories. Lot No BA10 - 18186.

Anti-H C5 (1) - Goat anti-human C5:

Kent Laboratories. Lot No 1100 N1. Anti-H C5 (2) - Sheep

Anti-human C5:

Seward Laboratories. Lot No BA12 - 1653A.

APPENDIX 2BUFFERS

1. Phosphate buffered saline (PBS). pH = 7.40

8000mg NaCl

200mg KCl or 10 PBS tablets

200mg KH_2PO_4 (Flow Laboratories)1442mg $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

The volume was made up to 1 litre with distilled water, autoclaved and stored at 4°C.

This was used for all PMN washing and counting procedures to prevent spurious degranulation.

2. Phosphate buffered saline with added calcium, magnesium and bovine serum albumin (PBS-A). pH = 7.40.

8000mg NaCl

200mg KCl or 10 PBS tablets

200mg KH_2PO_4 1442mg $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

The volume was made up to approximately 900mls with distilled water. To prevent formation of precipitates of calcium or magnesium phosphate, 1ml of 1.0M CaCl_2 and 0.7ml 1.0M MgCl_2 were each diluted in 10mls distilled water and added dropwise to the PBS in a mixer. The solution was autoclaved at this stage. One gram of cobalamin-binding protein-free bovine serum albumin was then added, and when dissolved, the solution was made up to 1 litre with distilled water and stored at 4°C.

Final concentrations of: calcium = 1 millimolar

magnesium = 0.7 millimolar

BSA = 0.1%

This solution was used for all degranulation studies.

3. Coupling buffer. (for anti-C₅ column preparation)

8.401g NaHCO₃ (final conc = 0.1M)

29.22g NaCl (final conc = 0.5M)

made up to 1 litre with distilled water. The pH was adjusted to 8.3.

4. Acetate buffer.

4.7mls glacial acetic acid

2.45g sodium acetate

58.44g sodium chloride (final conc = 1.0M)

made up to 1 litre with distilled water. The pH was adjusted to 4.0.

5. Borate buffer

6.183g boric acid

58.44g sodium chloride (final conc = 1.0M)

made up to 1 litre with distilled water. The pH was adjusted to 8.0 with strong sodium hydroxide.

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